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(54) Titre : EXTRAIT D'ALGUE ET INHIBITEUR DE LIPASE CONTENANT UN TEL EXTRAIT
(54) Title: ALGA EXTRACT AND LIPASE INHIBITOR CONTAINING THE SAME

(57) Abrégé/Abstract:
A lipase inhibitor characterized by containing, as the active ingredient, an extract of Ascophyllum nodsum belonging to brown algae can be employed as a useful health food or food for specified heal uses aiming at treating and preventing obesity or hyperlipemia.

ABSTRACT

A lipase inhibitor comprising as an active ingredient an extract of Ascophyllum nodosum which is a kind of brown algae can be used as a useful healthy food or food for specified health uses for the treatment and/or prevention of obesity or hyperlipemia.

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SPECIFICATION

MARINE ALGAE EXTRACT AND LIPASE INHIBITOR CONTAINING THE SAME

TECHNICAL FIELD

The present invention relates to a lipase inhibitor containing a marine algae extract as an active ingredient, more particularly, an extract of Ascophyllum nodosum which is a kind of brown algae, and also relates to a food and drink for the treatment and/or prevention of obesity and hyperlipemia.

BACKGROUND ART

Recently, with westernization of eating habits, obesity is increasing due to hypernutrition and the like. Obesity is one of risk factors of arteriosclerosis and concerned also with diabetes, hypertension and the like, thus, which has become a serious social problem. Obesity is a condition where fats are accumulated in excess in the body, and one of the causes is an excessive intake of fats.

In general, an excessive intake of calories works to increase stored calories, and as a result, stored calories increase in the body. That is, excessive intake of a fat of highest calorie among food components leads to obesity. Then, it is believed that obesity can be prevented or ameliorated by inhibiting a pathway from fat intake to obesity.

Fat in foods is not absorbed in its original form from the intestinal canal. Namely, the fat (triglyceride) is degraded by pancreatic lipase into fatty acid, and 2-monoglyceride or glycerol, which are then absorbed from

the intestine. In the intestine, these are resynthesized and transported into blood. Therefore, degradation of fat is suppressed, and absorption of fat from the intestinal canal is also suppressed, by inhibiting the activity of pancreatic lipase.

As such a lipase inhibitor, Orlistat (trade name: Zenical, Roche) is practically used abroad clinically as a medicine (see, e.g., non-patent literature 1: Ikeda Yoshio, "Recent use rate of Orlistat abroad", Himan Kenkyu, Nippon Obesity Institute, 2001, vol. 7, no. 3, p. 316-318).

However, this product is currently not accepted to be used in Japan, and cannot be used not only as a medicine but also as a food under the present situation.

A lipase inhibitor and a food containing this substance are useful for a patient suffering from related dysbolism since it can ameliorate pathological condition of the above-mentioned disease, and further, it is suitable also for prevention of obesity and hyperlipemia by taking into daily eating habits. Therefore, as a highly safe natural substance which is edible, there have hitherto been suggested lipase inhibitors derived from marine algae (see, e.g., patent literatures 1, 2 and 3: JP-A-5-284937, JP-A-10-203974, and JP-A-2000-236846), green pepper, pumpkin, shimeji mushroom, Glifola frondosa, Hizikia fusiforme, others (see, e.g., patent literature 4: JP-A-3-219872), Labiatae (see, e.g. patent literature 5: JP-A-10-262606), hop (see, e.g., patent literature 6: JP-A-2001-321166), defatted rice bran (see, e.g., patent literature 7: JP-A-2001-97880), tamarind seed coat (see, e.g., patent literature 8: JP-A-9-291039) and the like.

On the other hand, Ascophyllum nodosum is a marine algae belonging to brown algae, Fucales, Fucaceae, and mainly inhabits on the shore reef of ria shoreline in Norway. Ascophyllum nodosum is used as a raw material for producing alginic acid, because it contains alginic acid in high concentration. In addition, since Ascophyllum nodosum contains abundantly minerals and vitamins, a product obtained by drying of a raw alga and pulverization thereof is widely used as a feedstuff or a fertilizer and/or a soil improving agent. However, an extract of Ascophyllum nodosum has not been known to have an inhibitory action on lipase activity.

DISCLOSURE OF THE INVENTION

It is an object of the present invention to provide a lipase inhibiting substance derived from natural products, which inhibits pancreatic lipase as an enzyme involved in digestion and absorption of fats in an organism to suppress accumulation of fats in the body, and to provide a lipase inhibitor containing the above inhibiting substance.

The present inventors have intensively studied to solve the above-mentioned problem, and as a result, have found that an extract of Ascophyllum nodosum which is a kind of brown algae has a strong inhibitory action on pancreatic lipase, and further have found that this extract has also an action of lowering the plasma triglyceride, namely, has an action of decreasing the amount of triglyceride in plasma as well. The present inventors have further studied based on these findings, and completed the present invention.

That is, the present invention relates to:

(1) a lipase inhibitor comprising an extract of Ascophyllum nodosum as an active ingredient,

(2) a lipase inhibitor comprising a purified substance of the extract according to the above (1) as an active ingredient,

(3) the lipase inhibitor according to the above (1) or (2), which is in the form of food and drink,

(4) the lipase inhibitor according to the above (3), which is in the form of healthy food or food for specified health uses for the treatment and/or prevention of obesity,

(5) the lipase inhibitor according to the above (3), which is in the form of healthy food or food for specified health uses for the treatment and/or prevention of hyperlipemia,

(6) a plasma triglyceride-lowering agent comprising an extract of Ascophyllum nodosum as an active ingredient,

(7) a method of inhibiting lipase activity, which comprises administering an extract of Ascophyllum nodosum to a mammal,

(8) a method of treating and/or preventing obesity or hyperlipemia, which comprises administering an extract of Ascophyllum nodosum to a mammal,

(9) a method of lowering plasma triglyceride, which comprises administering an extract of Ascophyllum nodosum to a mammal,

(10) use of an extract of Ascophyllum nodosum for producing a medicine or food and drink which inhibits lipase activity,

(11) use of an extract of Ascophyllum nodosum for producing a medicine or food and drink for treating and/or preventing obesity or hyperlipemia, and

(12) use of an extract of Ascophyllum nodosum for producing a medicine or food and drink which lowers plasma triglyceride.

EFFECT OF THE INVENTION

The extract from Ascophyllum nodosum obtained in the present invention has a strong lipase inhibiting action and a strong plasma triglyceride lowering active action. Thus, a lipase inhibitor or triglyceride lowering agent comprising the above-mentioned extract can treat and/or prevent obesity and hyperlipemia more effectively as compared with conventionally known lipase inhibiting substances derived from marine algae.

The lipase inhibitor of the present invention can treat and/or prevent obesity and hyperlipemia. Thus, the lipase inhibitor is useful for patients suffering from dysbolism related to obesity and hyperlipemia, and can be incorporated in food and drink, particularly in healthy food or food for specified health uses in daily eating habits.

BEST MODES FOR CARRYING OUT THE INVENTION

In the present invention, any tissues and portions of Ascophyllum nodosum (hereinafter, abbreviated as Ascophyllum), preferably leaf and stem parts of algae can be used. In extracting from Ascophyllum, total algae or leaf

and stem parts of Ascophyllum harvested from the sea can be used as they are, or they can be cut, finely cut or ground, or dried them. Furthermore, total algae or leaf and stem parts of algae which is cut, finely cut or grounded after drying can be used. Preferably, total algae or leaf and stem parts of raw Ascophyllum which is grounded after drying can be used. Drying may be carried out by any methods known per se, for example, air drying, sun drying, freeze drying and the like.

As the extraction solvent, water or organic solvents, or

mixed solutions thereof are used. Examples of the organic solvent include polar organic solvents such as lower alcohols having 1 to 4 carbon atoms such as methanol, ethanol, propanol, isopropanol, n-butanol, isobutanol, sec-butanol, tert-butanol and the like, and ketones such as dimethyl ketone, methyl ethyl ketone, acetone, methyl isobutyl ketone and the like; and non-polar organic solvents such as methyl acetate, ethyl acetate, butyl acetate, diethyl ether and the like. These polar organic solvents and non-polar organic solvents can also be used in appropriate combination.

Of these extraction solvents, preferrable are polar organic solvents or mixed solutions of polar organic solvents and water, more preferable are methanol, ethanol or acetone or mixed solutions of them and water, and particularly preferable are mixed solutions of methanol, ethanol or acetone and water. The mixing ratio of a polar organic solvent to water varies depending on the kind of a polar organic solvent, and usually, polar organic solvent/water is in a range of about 5/95 to 100/0 (v/v). For example, when a methanol-water mixed solution or ethanol-water mixed solution is used as the extraction solvent, the ratio is about 5/95 to 100/0 (v/v), preferably about 30/70 to 70/30 (v/v). When an acetone-water mixed solution is used, the ratio is about 5/95 to 100/0 (v/v), preferably about 30/70 to 80/20 (v/v). These ratios are preferably determined taking extraction efficiency, amount of extracted substance, enzyme inhibitory activity of extracts, and the like into consideration.

In the present invention, the extraction method for obtaining an extract is not particularly restricted, and methods known per se can be used such as, for example, immersion

extraction, heat extraction, continuous extraction, supercritical extraction and the like. The ratio of Ascophyllum to extraction solvent is not particularly limited, and the ratio of dried Ascophyllum substance/solvent is preferably about 1/100 to 1/2 (w/v), more preferably about 1/10 to 1/5 (w/v). Specifically, extraction is preferably carried out while gently stirring or allowing to stand using an extraction solvent in an amount of about 200 mL to 10 L, preferably about 500 mL to 1 L based on about 100 g of the extraction raw material which is obtained by, for example, drying and grinding Ascophyllum. It is convenient in view of operability that the extraction temperature is in a range from room temperature to not higher than the boiling point of the solvent under normal pressure, and the extraction time varies depending on the extraction temperature and the like, and is in a range from several minutes to about 7 days, preferably from about 30 minutes to 24 hours.

After completion of the extraction operation, solid (extraction residue) is removed by methods known per se such as filtration, centrifugation and the like, to obtain an extract. The extract is concentrated by a method known per se, thereby to obtain an extract concentrated in the form of black to brown oil or paste (hereinafter, referred simply to as concentrate in some cases). The extract or the concentrate can also be converted into a solid extract by performing drying known per se such as, for example, thermal drying, freeze drying and the like. An extract, concentrate, or solution obtained by dissolving the concentrate in water and/or organic solvent may be purified by a method such as, for example, ultrafiltration, adsorption resin treatment, molecule chromatography, partition

chromatography, liquid-liquid extraction and the like. The purified extract can be used for the present invention in the form of purified substance.

The extract according to the present invention is useful as a lipase inhibitor since it has a strong lipase inhibitory action.

Regarding the lipase inhibitor of the present invention, it is preferable that the above-mentioned extract or purified substance is used as it is, or a pharmaceutically acceptable additive, or a food material, food raw material, further if necessary, a food additive and the like are appropriately mixed with the extract or purified substance, and they are preferably formulated into dosage form such as liquid, powder, granule, tablet, microcapsule, soft capsule, hard capsule and the like by methods known per se. Moreover, it is possible to make into any food and drink forms such as solid food, semisolid food like cream or jam, food like gel, beverage and the like. Examples of such food and drink include refreshing beverage, coffee, tea, milk-contained beverage, lactic acid bacteria beverage, drop, candy, chewing gum, chocolate, gummy candy, yoghurt, ice cream, pudding, soft adzuki-bean jelly, jelly, cookie and the like. These various preparations or foods and drinks are useful as a healthy food or food for specified health uses for the treatment and/or prevention of diabetes.

As the additive, food material, food raw material and food additive used in the production of the above-mentioned preparations or foods and drinks, for example, excipients (lactose, corn starch, white sugar, glucose, starch, crystalline cellulose and the like), lubricants (magnesium stearate,

sucrose fatty acid ester and the like), disintegrators (starch, carmellose sodium, calcium carbonate and the like), binders (starch paste liquid, hydroxypropylcellulose liquid, gum Arabic liquid and the like), emulsifiers and/or solubilizers (gum arabic, polysorbate 80, povidone and the like), sweeteners (white sugar, fructose, simple syrup, honey and the like), coloring agents (edible tar pigment, iron oxide and the like), preservatives (methyl p-oxybenzoate, propyl p-oxybenzoate, sorbic acid and the like), thickeners (hydroxyethylcellulose, polyethylene glycol, sodium alginate and the like), antioxidants (sodium hydrogen sulfite, sodium edetate, ascorbic acid and the like), stabilizers (sodium thiosulfate, sodium edentate, sodium citrate and the like), acidulants (lemon juice and the like), seasonings (sodium glutamate and the like), aromatics (mint, strawberry aroma and the like), and the like can be used.

The addition amount of the above-mentioned extract or purified substance based on the above-mentioned various preparations or foods and drinks is not uniform and varies depending on the content of a lipase inhibiting component contained in the extract or purified substance, and the amount of the extract (calculated as the solid) is, for example, about 0.0001 to 50 wt%, preferably about 0.001 to 20 wt%, more preferably about 0.01 to 10 wt%.

When these various preparations or foods or drinks are taken orally, the daily dose of the above-mentioned extract or purified substance is about 0.01 to 1000 mg, preferably about 0.1 to 500 mg, more preferably about 1 to 300 mg relative to 1 kg of body weight when calculated as the solid. This dose may be advantageously taken in one time or several times per day.

However, actual dose should be determined in view of the object and conditions of a person who takes it (sex, age, body weight, BMI and the like).

Preferable Examples in the present invention are described below, but the present invention is not limited to these Examples.

Example 1

About 50.0 g of a dried Ascophyllum powder was precisely weighed and to this dried powder was added 500 mL of an ethanol-water mixed solution in a ratio shown Table 1, and extraction was performed for 1 hour at room temperature with gentle stirring. The extraction solution was moved to a centrifuge tube, and divided into a supernatant and a precipitate by centrifugation, and 500 mL of the same ethanol-water mixed solution as described above was added to the precipitate, and extraction was performed for 1 hour in the same manner as in the first operation. The extract was divided into a supernatant and a precipitate in the same manner as in the first operation, and the supernatants of the first and second operations were combined and filtrated under suction, to obtain an extract in a total volume of about 1 L as a filtrate. This extract was concentrated at about 60°C under reduced pressure using a rotary evaporator, and the concentrate was freeze-dried to obtain extracts 1 to 6 in the form of black brown powder. The yields are shown in Table 1.

Table 1

Extract	Ethanol-water mixed solution (ethanol:water (v/v))	Yield (% by mass)
1	10:90	24.2
2	20:80	24.3
3	30:70	24.3
4	50:50	22.0
5	70:30	17.0
6	100:0	2.2

Example 2

To about 800 g of a dried Ascophyllum powder was added 8 L of an ethanol-water (50:50 (v/v)) mixed solution, and extraction was performed for 1 hour at room temperature with gentle stirring. The extract was moved to a centrifuge tube, and divided into a supernatant and a precipitate by centrifugation, and 8 L of the ethanol-water mixed solution was added to the precipitate, and extraction was performed for 1 hour in the same manner as in the first operation. The extract was divided into a supernatant and a precipitate in the same manner as in the first operation, and the supernatants of the first and second operations were combined and filtred under suction, to obtain an extract in a total volume of about 16 L as a filtrate. This extract was subjected to ultrafiltration using an ultrafiltration membrane having a fractional molecular weight of 10,000 (trade name: FB02-VC-FUSO181; Daicen Membrane Systems), and when the concentrated solution reached a volume of 5 L, 5 L of water was added thereto and filtration was continued, and when the concentrated solution reached again a volume of 5 L, ultrafiltration was stopped. The concentrated solution was concentrated at about 60°C under reduced pressure using a rotary evaporator, and the concentrate was freeze-dried to obtain

about 73 g of an extract (extract 7) in the form of black brown powder.

Example 3

The lipase inhibitory activity of the extract 7 obtained in Example 2 was measured using triolein as a substrate.

1) Measurement of lipase inhibiting activity

Sample solutions (1 mL each) containing the extract 7 in an amount of 5, 10, 50, 100 and 500 μg/mL respectively, 1 mL of 1 mg/mL lipase (Type II; Sigma) solution (pH 7.4), 7 mL of Mcilvaine buffer solution (pH 7.4), 100 mg of gum arabic, and 1.0 mg of triolein (manufactured by Wako Pure Chemicals Industries Ltd.) were mixed, and shaken at about 37°C for 1 hour, and 20 mL of ethanol was added to stop the reaction, thereby to obtain the reaction solution. In control group 1, 1 mL of Mcilvaine buffer solution (pH 7.4) was added instead of the lipase solution, and in control group 2, 1 mL of Mcilvaine buffer solution (pH 7.4) was added instead of the sample solution. To the reaction solution were added several drops of a phenolphthalein solution, and the reaction solution was titrated with 0.05 N NaOH, and the lipase inhibition rate was calculated according to the following equation.

Lipase inhibition rate (%) = (C-S)/(C-B) × 100

S: titration amount in test sample group (mL)

B: titration amount in control group 1 (mL)

C: titration amount in control group 2 (mL)

The lipase inhibition rate of the extract was measured and the results are shown in Table 2.

Table 2

Concentration of extract 7 (μg/mL)	Lipase inhibition rate (%)
5	9.2
10	15.4
50	67.7
100	80.0
500	90.8

The 50% inhibitory concentration (IC_{50}) of the extract 7 in Example 2 on lipase activity was found to be about 29 μg/mL which was calculated based on the above results.

Example 4

About 50.0 g of dried powders of various marine algae were precisely weighed and to these dried powders was added each 500 mL of an ethanol-water (30:70 (v/v)) mixed solution. The mixture was extracted for 1 hour at room temperature with gentle stirring. The extract was transferred to a centrifuge tube, and divided into a supernatant and a precipitate by centrifugation. 500 mL of the ethanol-water (30:70 (v/v)) mixed solution was added to the precipitate, and the mixture was extracted with for 1 hour in the same manner as in the first operation. The extract was divided into a supernatant and a precipitate in the same manner as in the first operation, and the supernatants of the first and second operations were combined and filtered under suction, thereby to obtain an extract in a total volume of about 1 L as a filtrate. This extract was concentrated at about 60°C under reduced pressure using a rotary evaporator, and the concentrate was freeze-dried to obtain extracts (extract 8, Comparative Examples 1 to 8) in the form of powder.

The lipase inhibition activity of these extracts was measured according to Example 3 described above. The

concentration of the sample solution was 100 μg/mL or 1000 μg/mL. The results are shown in Table 3 in terms of inhibition rate (%).

Table 3

	Kind of marine algae	Lipase inhibition rate (%)	
		100 μg/mL	1000 μg/mL
Extract 8	Ascophyllum nodosum (brown algae)	40.6	96.9
Comparative Example 1	Ulva pertusa Kjellman (green algae)	0	0
Comparative Example 2	Nemacystus decipiens (brown algae)	0	0
Comparative Example 3	Laminaria japonica (brown algae)	0	34.3
Comparative Example 4	Eisenia bicyclis (Kjellman) Setchell (brown algae)	7.2	25.0
Comparative Example 5	Undaria pinnatifida (brown algae)	4.5	20.0
Comparative Example 6	Sargassum fulvellum (brown algae)	5.8	27.1
Comparative Example 7	Hizikia fusiformis (brown algae)	0	0
Comparative Example 8	Ptilophora subcostata (red algae)	0	0

From Table 3, it is shown that an extract of Ascophyllum has a stronger lipase inhibition activity as compared with other marine algae, and additionally, the inhibitory activity is exhibited even at lower concentrations.

Example 5

Single administration test of oil was performed in mice using the extract 7 obtained in Example 2 as a sample.

1) Measurement of blood triglyceride lowering activity

Each eight 7-week old ddY mice fasted overnight were used in a control group and a sample administration group. About

50 μL of blood was collected from the mouse tail vein into a heparin-containing blood collection tube. After blood collection, an emulsion of olive oil and physiological saline so prepared as to give olive oil 5 g/body weight kg/6 mL was orally administered using a gastric tube, in the control group. Emulsions of olive oil and a sample solution dissolved in physiological saline so mixed and prepared that the olive oil was 5 g/body weight kg/6 mL and the extract 7 as a sample was 100 mg/body weight kg/6 mL or 500 mg/body weight kg/6 mL, were orally administered using a gastric sonde, in the sample administration group. About 50 μL of blood was collected 1, 2, 3, 4 and 5 hours after administration. The collected blood was centrifuged to fractionate a plasma fraction, and preserved at -40°C until analysis.

The plasma level of triglyceride was measured by GPO/DASO method using a measuring kit (Triglyceride E - Test Wako; manufactured by Wako Pure Chemical Industries Ltd.). The change with time of the plasma triglyceride level is shown in Table 4.

Table 4

Elapsed time (hour)	Plasma level of triglyceride (mg/100 mL) (numerical value is average value ± standard deviation)		
	Control group	Extract 7 administration group	
		100 mg/body weight kg	500 mg/body weight kg
0	336 ± 103	258 ± 62	267 ± 87
1	550 ± 147	514 ± 76	255 ± 81 **
2	1000 ± 284	781 ± 189	358 ± 126 **
3	1062 ± 300	747 ± 215 *	265 ± 83 **
4	78 ± 417	652 ± 229	180 ± 59 **
5	451 ± 356	413 ± 264	147 ± 26 **

** significant difference with a crisis ratio of 1% for the control group

* significant difference with a crisis ratio of 5% for the control group

It was found that an extract of Ascophyllum suppresses significantly increase in the plasma level of triglyceride 1 to 5 hours after administration as compared with the control group.

Example 6

To 50 parts by mass of lactose, 38 parts by mass of corn starch, 1 part by mass of strawberry aroma and 1 part by mass of sucrose fatty acid ester were added 10 parts by mass of the extract 7 in Example 2 and they were mixed, and then the mixture was tabletted using a tabletting machine to produce a supplement.

Example 7

A beverage solution having the composition shown in Table 5 was heated at about 65°C for 10 minutes, cooled to room

temperature, and filled aseptically in a sterile container to produce a honey lemon beverage.

Table 5

Component	Addition amount (% by mass)
Fructose-glucose syrup	11
Lemon juice	3
Honey	1
Aroma	0.1
Acidulant	0.1
Vitamin C	0.02
Pigment	0.01
Extract 7 of Example 2	1.00
Water	84.77
Total	100

Example 8

Orange jelly was produced according to the following procedure.

(1) 15 g of gelatin powder is placed in about 45 mL of water and swollen.

(2) 750 mL of orange juice (fruit juice 100%) and 90 g of granulated sugar are put in a pan and boiled. When the granulated sugar is dissolved, fire is extinguished, and about 7 g of the extract 7 in Example 2 and (1) are added and dissolved sufficiently.

(3) Ice water is allowed to contact the bottom of the pan and the content is cooled while mixing until thickened, and poured into a jelly mold of which inside wall has been moistened, and cooled to solidify.

INDUSTRIAL APPLICABILITY

An extract from Ascophyllum nodosum obtained in the present invention has a strong lipase inhibition action

and a strong plasma triglyceride lowering action. Therefore, a lipase inhibitor containing the extract from Ascophyllum nodosum can be used for more effective treatment and/or prevention of obesity and hyperlipemia as compared with conventionally known lipase- inhibiting substances derived from marine algae. Moreover, a food and drink containing the above-mentioned extract is useful as a healthy food or food for specified health uses for the treatment and/or prevention of obesity and hyperlipemia.

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CLAIMS:

1. A lipase inhibitor comprising an extract of Ascophyllum nodosum as an active ingredient.

2. A lipase inhibitor comprising a purified substance of the extract according to claim 1 as an active ingredient.

3. The lipase inhibitor according to claim 1 or 2, which is in the form of food and drink.

4. The lipase inhibitor according to claim 3, which is in the form of healthy food or food for specified health uses for the treatment and/or prevention of obesity.

5. The lipase inhibitor according to claim 3, which is in the form of healthy food or food for specified health uses for the treatment and/or prevention of hyperlipemia.

6. A plasma triglyceride-lowering agent comprising an extract of Ascophyllum nodosum as an active ingredient.

7. A method of inhibiting lipase activity, which comprises administering an extract of Ascophyllum nodosum to a mammal.

8. A method of treating and/or preventing obesity or hyperlipemia, which comprises administering an extract of Ascophyllum nodosum to a mammal.

9. A method of lowering plasma triglyceride, which comprises administering an extract of Ascophyllum nodosum to a mammal.

10. Use of an extract of Ascophyllum nodosum for producing a medicine or food and drink which inhibits lipase activity.

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11. Use of an extract of Ascophyllum nodosum for producing a medicine or food and drink for treating and/or preventing obesity or hyperlipemia.

12. Use of an extract of Ascophyllum nodosum for producing a medicine or food and drink which lowers plasma triglyceride.

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The requested image section for patent number 2548899 is unavailable. Cover pages and abstracts for patents registered before 1975 generally are not available.

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Journal of Applied Phycology 1: 319–323, 1989.

Isolation and characterization of brown algal polyphenols as inhibitors of α-amylase, lipase and trypsin

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Abstract

Extracts of *Ascophyllum nodosum*, *Fucus serratus*, *F. vesiculosus* and *Pelvetia canaliculata* contain inhibitors of α-amylase, lipase and trypsin. The inhibitors were isolated and identified by ^{1}H NMR spectroscopy as polyphenols which have apparent molecular weights in the range from 30000 to 100000 daltons, as determined by ultra-filtration with Amicon membranes. These polyphenols account for the whole of the inhibitory activity in crude algal extracts. The compounds inhibit α-amylase and trypsin in an apparently non-competitive manner, when preincubated with the enzymes, and the inhibition is directly proportional to the concentration of the inhibitor. Starch protects α-amylase when added to the enzyme together with the inhibitors. Under this condition the effectiveness of the inhibitors is reduced ten-fold.

Introduction

As part of a survey of British marine algae for compounds with biological activity, extracts of thirty-six species were tested for the presence of inhibitors of α-amylase. Only two species gave positive results and of these the highest activity occurred in extracts of *Ascophyllum nodosum* (L.) Le Jol. (Barwell *et al.*, 1981). As this alga is used in both animal feedstuffs and in human 'health foods' it was considered of interest to investigate further the nature of the enzyme inhibitory activity in extracts of *A. nodosum*. In addition, some other brown algae which commonly occur in either the same or similar habitats, and therefore may be harvested together with *A. nodosum*, were investigated.

Materials and methods

Laminaria digitata (Huds.) Lamour., *L. hyperborea* (Gunn.) Fosl., *Ascophyllum nodosum* (L.) Le Jol, *Fucus serratus* L., *F. vesiculosus* L., and *Pelvetia canaliculata* (L.) Dcne et Thur., were collected at Finavarra, County Clare, Ireland, in September 1982.

Extraction of algae and isolation of enzyme inhibitors

Algae were dried at 60 °C and powdered. Dry alga (5 g) was extracted with ethanol in a Soxhlet apparatus for 6 h and the extract evaporated *in vacuo* at 50 °C. The residue was extracted with

20 ml water and the extract clarified by centrifugation. This aqueous extract was used to determine total inhibitory activity. For isolation of inhibitors, the extract was dialysed against water and the non-dialysable fraction freeze-dried. The solids obtained were then fractionated into an ethyl acetate and acetone soluble fraction as described by Geiselman & McConnell (1981). ^{1}H NMR spectra were obtained in acetone d_6 using a Brüker WH-270 spectrometer.

Assay of enzymes and inhibitors

α-Amylase (EC 3.2.1.1.), Sigma type VII-A, was assayed at pH 7 and 37 °C, with 2 μg enzyme protein and 10 mg ml^{-1} soluble starch, as described by Robyt & Whelan (1968), using maltose as the standard reducing sugar. Lipase (EC 3.1.1.3.), Sigma type VI, was assayed at pH 8.0 and 37 °C, with 50 μg enzyme protein and 25% (v/v) olive oil, as described for the lipase assay kit No. 800, supplied by Sigma London Ltd., Poole, UK. Trypsin (EC 3.4.21.4.), Sigma type III, was assayed at pH 8.2 and 37 °C, with 10 μg enzyme protein and 1 mM N-α-benzoyl-DL-arginine p-nitroanilide hydrochloride, as described by Erlanger *et al.* (1961).

For the assay of inhibitors, enzyme was preincubated, in the absence and presence of inhibitor, for 5 min at 37 °C, after which the substrate was added. Preliminary experiments established that, in the absence of inhibitor, there was no loss of enzyme activity. Determinations of enzyme activity were made within the range where there was a linear relationship between the measured activity and concentration of enzyme. One inhibitor unit was contained in the volume of inhibitor solution which gave 50% inhibition of enzyme activity. Results are given as mean ± s.e. mean with the number of observations. Kinetic experiments were carried out by measuring initial velocities (v) with five substrate concentrations (s), ranging from one quarter to four times the apparent Michaelis constant. Kinetic constants were obtained from Hanes plots (s/v against s) in which lines of best fit were obtained by linear regression.

Results

Inhibitory activity of algal extracts

Table 1 shows that extracts of *Ascophyllum nodosum*, *Fucus serratus*, *F. vesiculosus* and *Pelvetia canaliculata* inhibited α-amylase, lipase and trypsin. In contrast, extracts of *Laminaria digitata* and *L. hyperborea* did not inhibit these enzymes.

Table 2 shows that the compounds responsible for the inhibitory activity towards α-amylase, extracted from *A. nodosum*, *F. serratus*, *F. vesiculosus* and *P. canaliculata*, were retained within dialysis tubing. All of the inhibitory compounds (102 ± 5%, $n = 3$) in an extract of *A. nodosum* passed through an Amicon 100A membrane whilst they were all (97 ± 6%, $n = 3$) retained by an Amicon PM30 membrane. Similar results were obtained for inhibitor activity towards lipase and

Table 1. Inhibitor activity towards α-amylase, lipase and trypsin in extracts of some brown algae. One inhibitor unit gave 50% inhibition of enzyme activity under conditions described in Materials and methods.

Algal species	Inhibitor units per g dry weight algae towards:		
	α-amylase	Lipase	Trypsin
A. nodosum	40000	1400	8000
F. serratus	40000	1800	16000
F. vesiculosus	80000	3000	16000
P. canaliculata	40000	1400	8000
L. digitata	0	0	0
L. hyperborea	0	0	0

Table 2. Effect of dialysis upon the inhibitor activity towards α-amylase. One inhibitor unit gave 50% inhibition of enzyme activity under conditions described in Materials and methods. Values are mean ± s.e., $n = 4$.

Algal species	α-amylase inhibitor units in:	
	Non-dialysed extract	Dialysed extract
A. nodosum	8300 ± 400	9400 ± 300
F. serratus	10200 ± 500	10000 ± 500
F. vesiculosus	12500 ± 300	12500 ± 500
P. canaliculata	9000 ± 350	8700 ± 300

trypsin, and for the inhibitor activity of extracts of *F. serratus*, *F. vesiculosus* and *P. canaliculata* towards α-amylase, lipase and trypsin.

Isolation of inhibitors

Ethyl acetate and acetone fractions of the non-dialysable solids, isolated from *A. nodosum*, *F. serratus*, *F. vesiculosus* and *P. canaliculata*, yielded light-brown solids which were freely soluble in water. The ^{1}H NMR spectra of the two fractions, from each alga, were essentially the same and exhibited resonances from δ5.67 to δ6.31, which closely matched those reported by Geiselman & McConnell (1981) for non-dialysable polyphenols isolated from *A. nodosum* and *F. vesiculosus*. The nature of the spectra indicated that the polyphenols constituted the great majority of the isolated material. Inhibitory activity towards α-amylase, lipase and trypsin was exhibited by the material in both the ethyl acetate and acetone fraction from *A. nodosum*, *F. serratus*, *F. vesiculosus* and *P. canaliculata*. Table 3 shows results obtained with *A. nodosum*. It can be seen that the majority of the inhibitory activity exhibited by the algal extract was recovered as the sum of the activity in the ethyl acetate and acetone fractions. In contrast, the ethyl acetate and acetone fractions of the non-dialysable solids from both *L. digitata* and *L. hyperborea* were only very small, did not exhibit resonances in the region from δ5.0 to δ7.0 in their ^{1}H NMR spectra and also did not inhibit any of the three enzymes.

Table 3. Inhibitory activity towards α-amylase, lipase and trypsin in an extract of *A. nodosum* and the ethyl acetate and acetone fractions derived from it. One inhibitor unit gave 50% inhibition of enzyme activity under conditions described in Materials and methods. Values are mean ± s.e. of 4 determinations upon fractions from one isolation procedure.

Fraction	Inhibitor units per g dry weight alga towards:		
	α-amylase	Lipase	Trypsin
Dialysed extract	40000 ± 900	1400 ± 50	8000 ± 300
Ethyl acetate	33000 ± 200	780 ± 20	6600 ± 180
Acetone	7700 ± 500	470 ± 30	1800 ± 100
Ethyl acetate + acetone	40700	1250	8400

Characterization of the enzyme inhibition

α-Amylase, lipase and trypsin were preincubated with inhibitor, isolated from *A. nodosum*, for times which ranged from 1 to 10 min. Maximum inhibition of each enzyme occurred within 1 min. With a standard preincubation time of 5 min, the percentage inhibition of each enzyme was directly proportional to the amount of inhibitor present. When the inhibition was calculated in terms of enzyme-protein inhibited, by inhibitors from 1 g dry weight of algae, similar values were obtained. The values obtained, with inhibitor isolated from *A. nodosum*, were; 40 ± 1 mg for α-amylase, 35 ± 1 mg for lipase and 40.0 ± 2 mg for trypsin (n = 4).

Hanes plots for α-amylase and trypsin activity, in the absence and presence of inhibitor from *A. nodosum*, were characteristic of non-competitive inhibition, in that the inhibitor reduced the maximum velocity whilst the Michaelis constant was not altered. With α-amylase, maximum velocities were 193 μM min^{-1} in the absence and 96 μM min^{-1} in the presence of inhibitor, whilst the Michaelis constant was 320 μg ml^{-1} in both conditions. With trypsin, maximum velocities were 7.5 μM min^{-1} in the absence and 2.6 μM min^{-1} in the presence of inhibitor, whilst the Michaelis constants were 780 μM and 770 μM, respectively. The possibility that starch might protect α-amylase from inhibition was investigated with inhibitor from *A. nodosum*. An amount which gave 90% inhibition, when preincubated with α-amylase, produced no inhibition when it was added to the enzyme at the same time as the substrate. Under these latter conditions, the amount of inhibitor required to produce 50% inhibition was ten times greater than when the enzyme was preincubated with inhibitor.

Discussion

Results of this investigation confirmed the original observation of Barwell *et al.* (1981) that extracts of *Ascophyllum nodosum* inhibit α-amylase. In addition, the present study showed that lipase and trypsin are inhibited and that extracts of *Fucus serratus*, *F. vesiculosus* and *Pelvetia canaliculata* also inhibit these enzymes. The inhibitors were non-dialysable. Since non-dialysable polyphenols, which could act as enzyme inhibitors (Loomis, 1966), occur in brown algae, the procedure used by Geiselman & McConnell (1981) to isolate non-dialysable polyphenols from *A. nodosum* and *F. vesiculosus* was applied to our inhibitor-containing extracts. The ^{1}H NMR spectra of the non-dialysable compounds isolated by us closely matched those recorded by Geiselman & McConnell (1981) and the spectra indicated that the isolated solids were composed almost entirely of phenolic compounds. The inhibitory activity of the dialysed extracts was quantitatively maintained in the polyphenol-containing fractions. Therefore, it appears that the inhibitor activity, in extracts of *A. nodosum*, *F. serratus*, *F. vesiculosus* and *P. canaliculata*, is due to their content of non-dialysable polyphenols. These behaved, during ultra-filtration, like spherical molecules with molecular weights greater than 30000 and less than 100000 Daltons. The brown algal polyphenols are composed predominantly of phloroglucinol units and can occur as branched structures (Ragan & Jensen, 1977). However, they are not spherical molecules and, therefore, their behaviour during ultra-filtration would not accurately indicate their molecular weights.

Extracts of *Laminaria digitata* and *L. hyperborea* did not inhibit α-amylase, lipase and trypsin and did not yield detectable amounts of non-dialysable polyphenols. Polyphenols have been reported in *Laminaria* species but, when present, occurred at much lower levels than in species of the Fucaceae (Ragan & Jensen, 1977).

The polyphenols isolated in this study inhibited each of the three mammalian digestive enzymes tested. Kinetic experiments with α-amylase and trypsin demonstrated that the inhibitors reduced the maximum velocity but did not alter the Michaelis constant. This effect is obtained with both non-competitive reversible inhibitors and irreversible inhibitors. The essentially linear relationship between percent inhibition and inhibitor concentration showed that the inhibition could not be of the reversible type, which would yield a hyperbolic relationship. Under the experimental conditions used here, polyphenols would react with protein by formation of hydrogen bonds with carbonyl groups of the proteins' peptide bonds. A high molecular weight polyphenol could form large numbers of hydrogen bonds with a protein molecule, resulting in a stable complex and apparently irreversible inhibition (Loomis, 1974). The fact that a particular amount of the isolated polyphenol inhibited essentially the same amount of α-amylase, lipase and trypsin protein demonstrated the non-specific nature of the interaction between the polyphenols and enzymes used here. Indeed, the polyphenols may be expected to inhibit most enzymes and react with most other proteins (Loomis, 1974). The non-dialysable polyphenols from *A. nodosum* and some other brown algae have been shown to cause non-specific aggregation of human erythrocytes (Rogers & Loveless, 1985; Blunden *et al.*, 1986), presumably because one polyphenol molecule can react with protein at the cell surface of several erythrocytes.

A. nodosum, *F. serratus*, *F. vesiculosus* and *P. canaliculata* can be incorporated into both animal feed-stuffs and human 'health foods'. The inhibitory effect of their polyphenols upon mammalian digestive enzymes raises the question of their suitability. The non-specific nature of the interaction of polyphenols with proteins means that they will interact with dietary protein so that their inhibitory effect upon digestive enzymes would be less than that predicted by *in vitro* tests with a single enzyme. Probably, the amount consumed in 'health foods' is negligible with regard to enzyme inhibition. Supplementation of the feed of pigs and sheep with brown algal meals can enhance weight gain (Homb, 1961). However, there is a relatively low limit to the amount of supplement which may be incorporated before

weight gain becomes less than with unsupplemented feed. This may be due to inhibition of digestive enzymes by algal polyphenols resulting in impaired digestion of the bulk of the feed's constituents. Below these levels seaweed meal supplements probably fulfill the role for which they are intended, that is as a source of vitamins and minerals (Guiry & Blunden, 1980).

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Inhibition of pancreatic lipase *in vitro* by the covalent inhibitor tetrahydrolipstatin

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Tetrahydrolipstatin inhibits pancreatic lipase from several species, including man, with comparable potency. The lipase is progressively inactivated through the formation of a long-lived covalent intermediate, probably with a 1:1 stoichiometry. The lipase substrate triolein and also a boronic acid derivative, which is presumed to be a transition-state-form inhibitor, retard the rate of inactivation. Therefore, in all probability, tetrahydrolipstatin reacts with pancreatic lipase at, or near, the substrate binding or active site. Tetrahydrolipstatin is a selective inhibitor of lipase; other hydrolases tested were at least a thousand times less potently inhibited.

INTRODUCTION

Pancreatic lipase is the key enzyme of dietary triacylglycerol absorption. It acts at the surface of emulsified lipid droplets [1]. Under physiological conditions triacylglycerol droplets are emulsified by bile salts [2] and colipase anchors and stabilizes lipase at the interphase [3]. A number of inhibitors of pancreatic lipase have been so far described; many are surfactants. Alkyl and aryl boronic acid derivatives are selective, reversible inhibitors of pancreatic lipase, and are supposed to act at the active centre [4]. Lipstatin, isolated from *Streptomyces toxytricini* [5], and its derivative tetrahydrolipstatin are the first selective irreversible inhibitors of lipase.

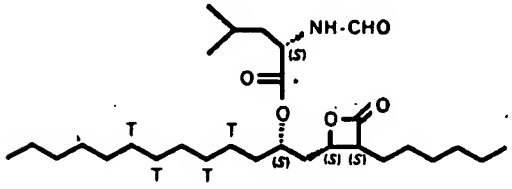
MATERIALS AND METHODS

Materials

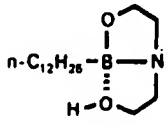
Tetrahydrolipstatin (M_r 496, see structure below) and [^{14}C]THL were obtained by chemical synthesis [6]. [^{3}H]THL was prepared from lipstatin, of microbial origin [7], by catalytic reduction with ^{3}H gas. The material obtained had a specific activity of 34 Ci/mmol and was diluted to 250 μCi/mmol for storage. It was repurified before use by t.l.c. (h.p.t.l.c silica gel plates from Merck, Darmstadt, Germany, developed with hexane:chloroform:dioxane, 1:1:0.22, by vol.). Ro 8-5263 (M_r 283.3) was synthesized by Professor Ramuz at F. Hoffmann-La Roche, Basle.

Trioleylglycerol ether was synthesized by a modification of the published method [8]. Porcine pancreatic lipase (EC 3.1.1.3) was purchased from Sigma (type VI-S, L 0382; lot 23-F-8095 was used in all but the experiment shown in Table 2, in which lot 55-F-8095 was used). An analysis performed by Professor B. Borgström (Lund, Sweden) showed that in comparison to his purified standard, only 28% of the protein of lot 23-F-8095 and only 14% of lot 55-F-8095 is active lipase (personal communication). Based on a total protein content of 70% and 66%, respectively, and an M_r of 50000, lot 23-F-8095 contains, maximally, 3.9 nmol, and lot 55-F-8095 1.85 nmol, of active lipase per mg. *N*-Terminal sequence analysis of lot 23-F-8095 showed that about 50% by weight of the protein corresponded to the sequence of lipase and the molar ratio of lipase to colipase was about 1.0:0.7. Human pancreatic juice was obtained from two patients after stimulation with cholecystokinin-pancreozymin. Intestinal fluid was recovered from four mice by rinsing the intestine with saline. α-Amylase (EC 3.2.1.1) from pig pancreas, esterase (EC 3.1.1.1) from pig liver, phospholipase A_2 (EC 3.1.1.4) from pig pancreas, chymotrypsin (EC 3.4.21.1) from bovine pancreas and trypsin (EC 3.4.21.4) from bovine pancreas were purchased from Boehringer Mannheim.

Succinyl-L-phenylalanine 4-nitroanilide was from SERVA, Heidelberg, Germany. Phosphatidylcholine (Soyaphosphatid NC 100) was obtained from Natermann, Köln, Germany, and sodium salts of bile acids were purchased from Calbiochem. All other chemicals were from FLUKA, Buchs, Switzerland.



THL (T: ^{3}H label, *: ^{14}C label)



Ro 8-5263

Abbreviation used: THL, tetrahydrolipstatin.

Methods

All experiments were performed at least three times and representative single experiments are shown.

Pancreatic lipase activity

For the determination of lipase activity, the hydrolysis of triolein to fatty acids was followed at pH 8 for 10 min at room temperature using a recording pH-stat (Metrohm, Herisau, Switzerland, modified for a 100 μl syringe). The substrate emulsion (1.5 ml per assay) was prepared by ultrasonication of triolein (30 mg/ml) in a solution containing 1 mM-taurochenodeoxycholate, 9 mM-taurocholate, 0.1 mM-cholesterol, 1 mM-phosphatidylcholine, 15 mg of bovine serum albumin/ml, 2 mM-Tris/HCl, 100 mM-NaCl and 1 mM-$CaCl_2$ [a Branson (Danbury, CT, U.S.A.) Sonifier was used]. After addition of the test compound dissolved in 150 μl of dimethyl sulphoxide, or vehicle alone, the pH was adjusted to 8.0 and the reaction was started within 1 min by the addition of 15–20 μl of lipase (dissolved in saline/4% bovine serum albumin at a concentration of 70 μg/ml) or 100 μl of duodenal juice. A standard lipolytic activity was achieved by adding sufficient lipase to result in the liberation of 0.2–0.3 μmol of fatty acids/min per ml.

Activity of additional hydrolases

α-Amylase activity (1 unit in 1.5 ml final volume) was measured for 5 min at 30 °C with soluble starch as substrate according to Bernfeld [9].

Esterase activity was measured with ethyl butyrate as substrate and quantification of the liberated ethanol with alcohol dehydrogenase/NAD^+ by a combination of the methods described for these enzymes [10].

Phospholipase A_2 activity was measured at room temperature with egg yolk (which contains phosphatidylcholine as the main phospholipid) as substrate by titration of the liberated fatty acids with a recording pH-stat set to pH 8.0, essentially as described in [10].

Chymotrypsin activity was measured with succinyl-L-phenylalanine 4-nitroanilide as substrate [11].

Trypsin activity was measured with benzoylarginine *p*-nitroanilide as substrate [12].

In all assays bovine serum albumin, 25 mg/ml, was included. THL was added in dimethyl sulphoxide (10% final concentration) and the reaction was started immediately by the addition of the enzyme.

RESULTS AND DISCUSSION

Inhibition of lipase activity

Determination of lipase activity was based on the amount of oleic acid liberated from emulsified glycerol trioleate. The composition of the test emulsion was chosen to mimick as closely as possible the conditions *in vivo*. The concentration of bile acids (10 mM) approximated concentrations found in duodenal aspirates [13], and phospholipids, cholesterol and protein were also included. The titration curves of the liberated fatty acids approached a slightly sigmoidal shape (Fig. 1*a*); this was probably due to modulation of the interphase by the generated products [14]. Especially in the presence of calcium, the substrate surface can undergo profound changes in the course of the lipolytic process [15]. Nevertheless, an almost linear dependence of lipolytic activity on the amount of lipase added was obtained (not shown). The degree of lipase inhibition by THL largely depended on the time of incubation with the enzyme. As THL is insoluble in the absence of the substrate, preincubation with the enzyme is impractical. Therefore, inhibitory activity was calculated from the activity measured during the first 10 min of incubation after addition of enzyme to substrate plus inhibitor. The resulting dose–response curve (Fig. 1*b*) for THL is suitable for comparative quantification of the inhibitory activities.

For an equal initial lipolytic activity, 50% inhibition resulted from the addition of 0.11 μg of THL/ml to porcine pancreatic lipase, of 0.27 μg/ml to mouse intestinal fluid and of 0.12 μg/ml to human duodenal juice. Not only is the amount of THL necessary for half-maximal inhibition of lipase activity from the three species very similar, but also the dose–response curves parallel each other (not shown). The octanol/buffer, pH 7.5, partition coefficient of THL is greater than 1000. In accordance, in our test system [^{3}H]THL is recovered exclusively in the lipids after phase separation by ultracentrifugation or ultrafiltration through a 0.2 μm membrane filter. Therefore, the concentration of THL for a given degree of inhibition is dependent on the lipid phase present and the calculation of a K_i value is meaningless. The lipolytic activity of the intestinal content can largely be attributed to pancreatic lipase, but some lingual (gastric) lipase activity may also be present [16]. Since THL completely inhibits the lipolytic activity of intestinal fluid, lingual lipase, if present and active on the substrate used, is also inhibited.

Selectivity of lipase inhibition

Of the hydrolases evaluated, only liver esterase was slightly inhibited at the highest concentrations of THL which could be tested (Table 1). High selectivity of THL for lipase is apparent since the concentration of THL necessary to inhibit esterase was three orders of magnitude higher than for lipase. Esterase and lipase have overlapping substrate specificities and probably related enzymic mechanisms; therefore, a slight inhibition of esterase by THL is not surprising.

Formation of a covalent lipase–THL complex

Binding experiments with [^{3}H]THL incubated for 10 min with the triolein emulsion and with a 10–50-fold molar excess of lipase over THL showed that over 90% of the radioactivity remained bound to the protein even after denaturation by extraction with chloroform/methanol [17]. If bovine serum albumin was substituted for lipase and subjected to the same procedure, then less than 1% of the radioactivity was recovered in the protein precipitate. This excludes an unspecific reaction of THL with proteins. After incubation of lipase as above, the addition of 1 M-HCl for 24 h at room temperature prior to the extraction did not affect the binding of THL to the lipase; in contrast, treatment with 1 M-NaOH for 1 h led to complete release of the radioactivity into the chloroform phase. Over 90% of the radioactivity from [^{14}C]THL labelled in the leucine part of the molecule was

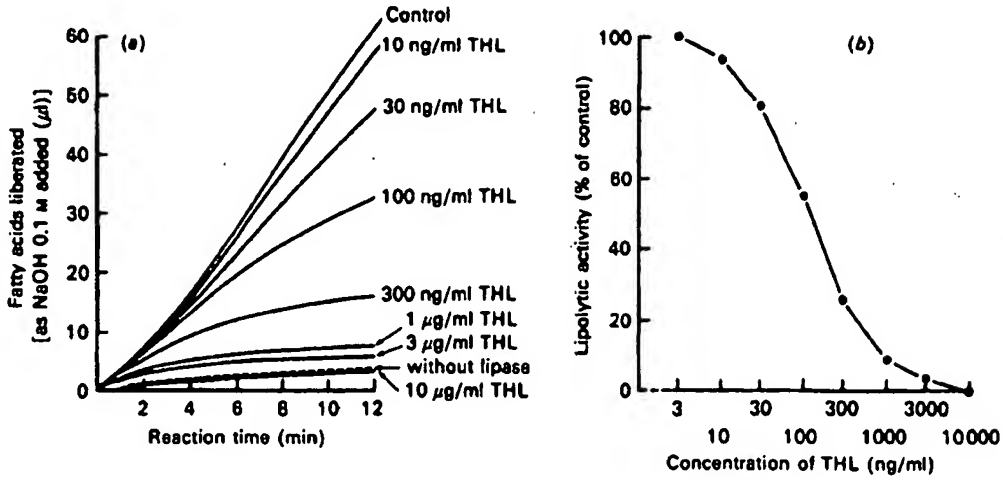


Fig. 1. Inhibition of pancreatic lipase by THL

(*a*) Titration curves for the release of oleic acid from triolein in the presence of different amounts of THL. (*b*) Inhibition of lipolytic activity as calculated from the amount of fatty acid released from $t = 0$ to $t = 10$ min compared with control at a given THL concentration. Data are derived from (*a*). For experimental details see under 'Materials and methods'.

also bound by lipase. In the presence of saturating amounts of [^{3}H]THL, incorporation of radioactivity by lipase was paralleled by loss of enzymic activity (Table 2). Because of the low percentage of active lipase in the available preparation, the stoichiometry of THL bound to lipase could not be exactly determined. Based on a content of 1.85 nmol of active lipase/mg of the preparation used in this experiment, it seems very probable that the binding of 1 molecule of THL per molecule of lipase is sufficient for complete inhibition of lipolytic activity. A stoichiometry close to 1:1 is supported by experimental results obtained both with an older lot (23-F-8095) of lipase from Sigma, which had twice the specific activity as compared with the lot shown in Table 2, and with a reference sample of purified porcine pancreatic lipase, a generous gift from Professor B. Borgström, Lund, Sweden.

Close analogues of THL that lack the β-lactone ring (e.g. the β-hydroxy acid analogue) are completely inactive (not shown). This suggests that the functional group of THL that is involved in covalent bond formation with lipase is the β-lactone. Since no reaction occurs with serum albumin and since the reaction stoichiometry with lipase is close to 1:1, it seems that this reaction takes place only at a specific microenvironment within the lipase molecule.

Table 1. Inhibition of various hydrolases by THL

The highest concentration of THL which could be obtained in the respective assay system was used and the residual enzyme activity at this concentration is shown.

Enzyme	Highest concn. of THL tested (μM)	Residual activity (%)
Trypsin	200	92
Chymotrypsin	200	79
Esterase (liver)	200	55
Phospholipase A_2	2000	100
Amylase	600	100

Rate of lipase inhibition

Lipase inhibition by THL is progressive with time and the rate of inactivation increases with increasing concentration of THL (see Fig. 2). After precipitation of lipase and removal of excess THL by extraction with acetone (67% final concentration, 0 °C), 50% reactivation occurs in buffer solution in 24 h. Hence, from a practical point of view, inhibition of pancreatic lipase by

Table 2. Binding of [^{3}H]THL to pancreatic lipase and loss of lipolytic activity

Crude porcine pancreatic lipase (500 μg) (Sigma no. L 0382, lot 55-F-8095) was preincubated in a final volume of 275 μl containing 125 μl of triolein emulsion (see under 'Methods') with the indicated amount of [^{3}H]THL (added in 25 μl of dimethyl sulphoxide) for 45 min at room temperature. Residual activity was measured by addition of 25 μl of the 1:10 diluted preincubation mixture to 1.5 ml of triolein emulsion. Unbound [^{3}H]THL was extracted with chloroform/methanol [17]. The calculated amount of active lipase in the preincubation mixture was 0.92 nmol (see under 'Materials').

Preincubation with [^{3}H]THL (nmol)	[^{3}H]THL bound to lipase (nmol)	Inhibition of lipolytic activity (%)
0.23	0.10	11
0.52	0.21	23
0.99	0.39	42
1.54	0.54	58
2.04	0.65	71
2.55	0.77	84
3.01	0.86	93

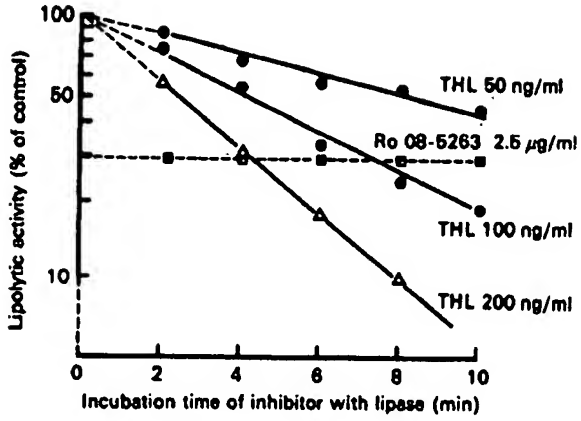


Fig. 2. Time dependence of the inhibition of pancreatic lipase by THL

Percentage inhibition achieved with three different concentrations of THL in the presence of substrate was derived from the slope of the titration curve at the indicated time points and expressed as a percentage of the slope of the control curve after hydrolysis of the same amount of triolein. The inhibition kinetics of a presumed competitive inhibitor, the boron compound Ro 8-5263, are shown for comparison.

THL is irreversible. It can be inferred that the putative covalent intermediate is relatively stable, although it is hydrolysed in buffer over a period of several hours (cf. its stability in acid and instability in alkali noted above). In the usual assay method for irreversible inhibitors [18,19], aliquots of the incubation mixture are diluted into the assay system in order to lower the inhibitor concentration sufficiently to stop the inactivation process. This was not applicable with our test system, because using this method the plot of log(enzyme activity remaining) versus time also gave straight lines but the extrapolated activity at zero time was substantially lower than 100% and dependent on the THL concentration. In contrast to THL, Ro 8-5263, a presumed competitive inhibitor of lipase, provoked a constant inhibition over time (Fig. 2). In analogy to other boronic acid inhibitors of serine esterases [20], the latter compound is expected to be a transition state form inhibitor of lipase, especially as the boron in Ro 8-5263 is tetrahedral as determined by X-ray crystallographic analysis. In the presence of Ro 8-5263 and dependent on its concentration the rate of covalent inhibition of pancreatic lipase by THL is slowed down (measured after 10-fold dilution of the preincubation mixture; not shown).

The substrate triolein strongly retards the rate of inactivation of lipase by THL, as shown in Fig. 3. Since THL is entirely distributed into the lipid phase, a constant concentration of this inhibitor could only be achieved by keeping the total lipid mass constant through substitution of a nonhydrolysable fat, trioleoylglycerol ether, for triolein. Preincubation of lipase with trioleoylglycerol ether in the presence of triolein is without major impact on lipolytic activity, whereas preincubation with the ether without substrate largely inactivates the enzyme. For these reasons preincubation of lipase with THL but without substrate was performed in the absence of a lipid phase but in this case THL was dissolved in the total volume of the sample and not concentrated in the lipid phase. In consequence the effective inhibitor concentrations are different in the two experiments and therefore the results obtained by preincubation without substrate are shown separately (Fig. 3*a*).

Since the inactivation rate of lipase by THL is slowed down by the substrate triolein and by a boronic acid derivative, which is presumed to be a transition-state-form inhibitor, it is probable that THL binds at or near the substrate binding or active site of lipase. Determination of the amino acid residue of pancreatic lipase to which THL is bound is in progress.

We thank Professor K. Gyr, Kantonsspital Basel, Switzerland, for providing us with human pancreatic juice. We are also grateful to our colleagues Dr. P. Barbier for the synthesis of THL, Professor H. Ramuz for providing us with Ro 8-5263, Dr. U. Widmer for synthesis of trioleoylglycerol

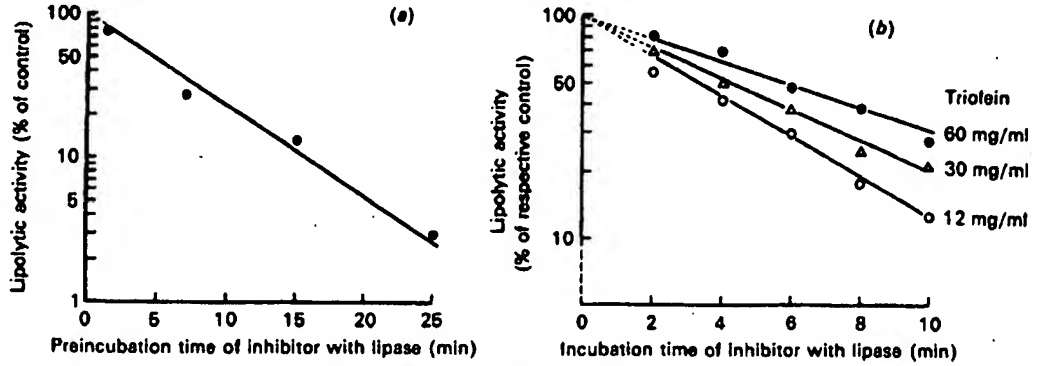


Fig. 3. Influence of substrate concentration on the progressive development of lipase inhibition produced by THL: *(a)* preincubation of lipase with THL without substrate, and *(b)* incubation of lipase with THL in the presence of varying amounts of substrate

(*a*) 0.6 μg of pancreatic lipase was preincubated with THL (100 ng/ml) in a final volume of 230 μl, containing 1% dimethyl sulphoxide. Initial rate of the remaining lipolytic activity (tangent to the titration curve of the liberated fatty acids) was measured after addition of 1.5 ml of triolein emulsion. (*b*) Percentage inhibition achieved with THL (100 ng/ml) was measured as described in Fig. 2. Since THL has a high preference for the lipid phase, the total lipid mass was kept constant (60 mg/ml) by substituting trioleylglycerol ether for triolein. As a result a constant concentration of THL in the lipid phase was achieved.

ether, Dr. H. P. Kocher for performing the *N*-terminal amino acid sequence analysis and Dr. N. Gains for editing the manuscript. The excellent secretarial work of Mrs. D. Brütsch is also gratefully acknowledged.

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XENICAL®
(orlistat)
CAPSULES

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XENICAL®

(orlistat)

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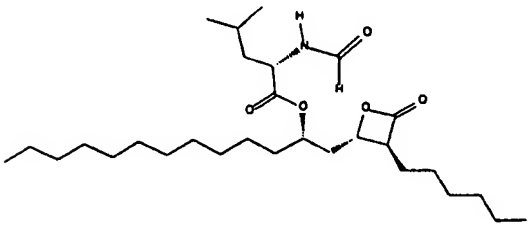
DESCRIPTION

XENICAL (orlistat) is a lipase inhibitor for obesity management that acts by inhibiting the absorption of dietary fats.

Orlistat is (S)-2-formylamino-4-methyl-pentanoic acid (S)-1-[[(2S, 3S)-3-hexyl-4-oxo-2-oxetanyl] methyl]-dodecyl ester. Its empirical formula is $C_{29}H_{53}NO_5$, and its molecular weight is 495.7. It is a single diastereomeric molecule that contains four chiral centers, with a negative optical rotation in ethanol at 529 nm. The structure is:



Orlistat is a white to off-white crystalline powder. Orlistat is practically insoluble in water, freely soluble in chloroform, and very soluble in methanol and ethanol. Orlistat has no pK_a within the physiological pH range.

XENICAL is available for oral administration in dark-blue, hard-gelatin capsules, with light-blue imprinting. Each capsule contains 120 mg of the active ingredient, orlistat. The capsules also contain the inactive ingredients microcrystalline cellulose, sodium starch glycolate, sodium lauryl sulfate, povidone, and talc. Each capsule shell contains gelatin, titanium dioxide, and FD&C Blue No.1, with printing of pharmaceutical glaze NF, titanium dioxide, and FD&C Blue No.1 aluminum lake.

CLINICAL PHARMACOLOGY

Mechanism of Action

Orlistat is a reversible inhibitor of lipases. It exerts its therapeutic activity in the lumen of the stomach and small intestine by forming a covalent bond with the active serine residue site of gastric and pancreatic lipases. The inactivated enzymes are thus unavailable to hydrolyze dietary fat in the form of triglycerides into absorbable free fatty acids and monoglycerides. As undigested triglycerides are not absorbed, the resulting caloric deficit may have a positive effect on weight control. Systemic absorption of the drug is therefore not needed for activity. At the recommended therapeutic dose of 120 mg three times a day, orlistat inhibits dietary fat absorption by approximately 30%.

Pharmacokinetics

Absorption

Systemic exposure to orlistat is minimal. Following oral dosing with 360 mg ^{14}C-orlistat, plasma radioactivity peaked at approximately 8 hours; plasma concentrations of intact orlistat were near the limits of detection (<5 ng/mL). In therapeutic studies involving monitoring of plasma samples, detection of intact orlistat in plasma was sporadic and concentrations were low (<10 ng/mL or 0.02 μM), without evidence of accumulation, and consistent with minimal absorption.

The average absolute bioavailability of intact orlistat was assessed in studies with male rats at oral doses of 150 and 1000 mg/kg/day and in male dogs at oral doses of 100 and 1000 mg/kg/day and found to be 0.12%, 0.59% in rats and 0.7%, 1.9% in dogs, respectively.

Distribution

In vitro orlistat was >99% bound to plasma proteins (lipoproteins and albumin were major binding proteins). Orlistat minimally partitioned into erythrocytes.

Metabolism

Based on animal data, it is likely that the metabolism of orlistat occurs mainly within the gastrointestinal wall. Based on an oral ^{14}C-orlistat mass balance study in obese patients, two metabolites, M1 (4-member lactone ring hydrolyzed) and M3 (M1 with N-formyl leucine moiety cleaved), accounted for approximately 42% of total radioactivity in plasma. M1 and M3 have an open β-lactone ring and extremely weak lipase inhibitory activity (1000- and 2500-fold less than orlistat, respectively). In view of this low inhibitory activity and the low plasma levels at the therapeutic dose (average of 26 ng/mL and 108 ng/mL for M1 and M3, respectively, 2 to 4 hours after a dose), these metabolites are considered pharmacologically inconsequential. The primary metabolite M1 had a short half-life (approximately 3 hours) whereas the secondary metabolite M3 disappeared at a slower rate (half-life approximately 13.5 hours). In obese patients, steady-state plasma levels of M1, but not M3, increased in proportion to orlistat doses.

Elimination

Following a single oral dose of 360 mg ^{14}C-orlistat in both normal weight and obese subjects, fecal excretion of the unabsorbed drug was found to be the major route of elimination. Orlistat and its M1 and M3 metabolites were also subject to biliary excretion. Approximately 97% of the administered radioactivity was excreted in feces; 83% of that was found to be unchanged orlistat. The cumulative renal excretion of total radioactivity was <2% of the given dose of 360 mg ^{14}C-orlistat. The time to reach complete excretion (fecal plus urinary) was 3 to 5 days. The disposition of orlistat appeared to be similar between normal weight and obese subjects. Based on limited data, the half-life of the absorbed orlistat is in the range of 1 to 2 hours.

Special Populations

Because the drug is minimally absorbed, studies in special populations (geriatric, different races, patients with renal and hepatic insufficiency) were not conducted.

Pediatrics

Plasma concentrations of orlistat and its metabolites M1 and M3 were similar to those found in adults at the same dose level. Daily fecal fat excretions were 27% and 7% of dietary intake in orlistat and placebo treatment groups, respectively.

Drug-Drug Interactions

Drug-drug interaction studies indicate that XENICAL had no effect on pharmacokinetics and/or pharmacodynamics of alcohol, digoxin, glyburide, nifedipine (extended-release tablets), oral contraceptives, phenytoin, pravastatin, or warfarin. Alcohol did not affect the pharmacodynamics of orlistat.

Other Short-term Studies

Adults

In several studies of up to 6-weeks duration, the effects of therapeutic doses of XENICAL on gastrointestinal and systemic physiological processes were assessed in normal-weight and obese subjects. Postprandial cholecystokinin plasma concentrations were lowered after multiple doses of XENICAL in two studies but not significantly different from placebo in two other experiments. There were no clinically significant changes observed in gallbladder motility, bile composition or lithogenicity, or colonic cell proliferation rate, and no clinically significant reduction of gastric emptying time or gastric acidity. In addition, no effects on plasma triglyceride levels or systemic lipases were observed with the administration of XENICAL in these studies. In a 3-week study of 28 healthy male volunteers, XENICAL (120 mg three times a day) did not significantly affect the balance of calcium, magnesium, phosphorus, zinc, copper, and iron.

Pediatrics

In a 3-week study of 32 obese adolescents aged 12 to 16 years, XENICAL (120 mg three times a day) did not significantly affect the balance of calcium, magnesium, phosphorus, zinc, or copper. The iron balance was decreased by 64.7 μmole/24 hours and 40.4 μmole/24 hours in orlistat and placebo treatment groups, respectively.

Dose-response Relationship

A simple maximum effect (E_{max}) model was used to define the dose-response curve of the relationship between XENICAL daily dose and fecal fat excretion as representative of gastrointestinal lipase inhibition. The dose-response curve demonstrated a steep portion for doses up to approximately 400 mg daily, followed by a plateau for higher doses. At doses greater than 120 mg three times a day, the percentage increase in effect was minimal.

CLINICAL STUDIES

Observational epidemiologic studies have established a relationship between obesity and visceral fat and the risks for cardiovascular disease, type 2 diabetes, certain forms of cancer, gallstones, certain respiratory disorders, and an increase in overall mortality. These studies suggest that weight loss, if maintained, may produce health benefits for obese patients who have or are at risk of developing weight-related comorbidities. The long-term effects of orlistat on morbidity and mortality associated with obesity have not been established.

The effects of XENICAL on weight loss, weight maintenance, and weight regain and on a number of comorbidities (eg, type 2 diabetes, lipids, blood pressure) were assessed in the 4-year XENDOS study and in seven long-term (1- to 2-years duration) multicenter, double-blind, placebo-controlled clinical trials. During the first year of therapy, the studies of 2-year duration assessed weight loss and weight maintenance. During the second year of therapy, some studies assessed continued weight loss and weight maintenance and others assessed the effect of orlistat on weight regain. These studies included over 2800 patients treated with XENICAL and 1400 patients treated with placebo. The majority of these patients had obesity-related risk factors and comorbidities. In the XENDOS study, which included 3304 patients, the time to onset of type 2 diabetes was assessed in addition to weight management. In all these studies, treatment with XENICAL and placebo designates treatment with XENICAL plus diet and placebo plus diet, respectively.

During the weight loss and weight maintenance period, a well-balanced, reduced-calorie diet that was intended to result in an approximate 20% decrease in caloric intake and provide 30% of calories from fat was recommended to all patients. In addition, all patients were offered nutritional counseling.

One-year Results: Weight Loss, Weight Maintenance, and Risk Factors

Weight loss was observed within 2 weeks of initiation of therapy and continued for 6 to 12 months.

Pooled data from five clinical trials indicated that the overall mean weight loss from randomization to the end of 6 months and 1 year of treatment in the intent-to-treat population were 12.4 lbs and 13.4 lbs in the patients treated with XENICAL and 6.2 lbs and 5.8 lbs in the placebo-treated patients, respectively. During the 4-week placebo lead-in period of the studies, an additional 5 to 6 lb weight loss was also observed in the same patients. Of the patients who completed 1 year of treatment, 57% of the patients treated with XENICAL (120 mg three times a day) and 31% of the placebo-treated patients lost at least 5% of their baseline body weight.

The percentages of patients achieving ≥5% and ≥10% weight loss after 1 year in five large multicenter studies for the intent-to-treat populations are presented in Table 1.

Table 1 Percentage of Patients Losing ≥5% and ≥10% of Body Weight From Randomization After 1-Year Treatment*

Intent-to-Treat Population†									
	≥5% Weight Loss					≥10% Weight Loss			
Study No.	XENICAL	n	Placebo	n	p-value	XENICAL	n	Placebo	n	p-value
14119B	35.5%	110	21.3%	108	0.021	16.4%	110	6.5%	108	0.022
14119C	54.8%	343	27.4%	340	<0.001	24.8%	343	8.2%	340	<0.001
14149	50.6%	241	26.3%	236	<0.001	22.8%	241	11.9%	236	0.02
14161‡	37.1%	210	16.0%	212	<0.001	19.5%	210	3.8%	212	<0.001
14185	42.6%	657	22.4%	223	<0.001	17.7%	657	9.9%	223	0.006

The diet utilized during year 1 was a reduced-calorie diet.

* Treatment designates XENICAL 120 mg three times a day plus diet or placebo plus diet

† Last observation carried forward

‡ All studies, with the exception of 14161, were conducted at centers specialized in treating obesity and complications of obesity. Study 14161 was conducted with primary care physicians.

The relative changes in risk factors associated with obesity following 1 year of therapy with XENICAL and placebo are presented for the population as a whole and for the population with abnormal values at randomization.

Population as a Whole

The changes in metabolic, cardiovascular and anthropometric risk factors associated with obesity based on pooled data for five clinical studies, regardless of the patient's risk factor status at randomization, are presented in Table 2. One year of therapy with XENICAL resulted in relative improvement in several risk factors.

Table 2 Mean Change in Risk Factors From Randomization Following 1-Year Treatment* Population as a Whole

Risk Factor	XENICAL 120 mg†	Placebo†
Metabolic:		
Total Cholesterol	-2.0%	+5.0%
LDL-Cholesterol	-4.0%	+5.0%
HDL-Cholesterol	+9.3%	+12.8%
LDL/HDL	-0.37	-0.20
Triglycerides	+1.34%	+2.9%
Fasting Glucose, mmol/L	-0.04	+0.0
Fasting Insulin, pmol/L	-6.7	+5.2
Cardiovascular:		
Systolic Blood Pressure, mm Hg	-1.01	+0.58
Diastolic Blood Pressure, mm Hg	-1.19	+0.46
Anthropometric:		
Waist Circumference, cm	-6.45	-4.04
Hip Circumference, cm	-5.31	-2.96

* Treatment designates XENICAL 120 mg three times a day plus diet or placebo plus diet

† Intent-to-treat population at week 52, observed data based on pooled data from 5 studies

Population With Abnormal Risk Factors at Randomization

The changes from randomization following 1-year treatment in the population with abnormal lipid levels (LDL ≥ 130 mg/dL, LDL/HDL ≥ 3.5, HDL <35 mg/dL) were greater for XENICAL compared to placebo with respect to LDL-cholesterol (-7.83% vs +1.14%) and the LDL/HDL ratio (-0.64 vs -0.46). HDL increased in the placebo group by 20.1% and in the XENICAL group by 18.8%. In the population with abnormal blood pressure at baseline (systolic BP ≥ 140 mm Hg), the change in SBP from randomization to 1 year was greater for XENICAL (-10.89 mm Hg) than placebo (-5.07 mm Hg). For patients with a diastolic blood pressure ≥90 mm Hg, XENICAL patients decreased by -7.9 mm Hg while the placebo patients decreased by -5.5 mm Hg. Fasting insulin decreased more for XENICAL than placebo (-39 vs -16 pmol/L) from randomization to 1 year in the population with abnormal baseline values (≥120 pmol/L). A greater reduction in waist circumference for XENICAL vs placebo (-7.29 vs -4.53 cm) was observed in the population with abnormal baseline values (≥100 cm).

Effect on Weight Regain

Three studies were designed to evaluate the effects of XENICAL compared to placebo in reducing weight regain after a previous weight loss achieved following either diet alone (one study, 14302) or prior treatment with XENICAL (two studies, 14119C and 14185). The diet utilized during the 1-year weight regain portion of the studies was a weight-

maintenance diet, rather than a weight-loss diet, and patients received less nutritional counseling than patients in weight-loss studies. For studies 14119C and 14185, patients' previous weight loss was due to 1 year of treatment with XENICAL in conjunction with a mildly hypocaloric diet. Study 14302 was conducted to evaluate the effects of 1 year of treatment with XENICAL on weight regain in patients who had lost 8% or more of their body weight in the previous 6 months on diet alone.

In study 14119C, patients treated with placebo regained 52% of the weight they had previously lost while the patients treated with XENICAL regained 26% of the weight they had previously lost ($p<0.001$). In study 14185, patients treated with placebo regained 63% of the weight they had previously lost while the patients treated with XENICAL regained 35% of the weight they had lost ($p<0.001$). In study 14302, patients treated with placebo regained 53% of the weight they had previously lost while the patients treated with XENICAL regained 32% of the weight that they had lost ($p<0.001$).

Two-year Results: Long-term Weight Control and Risk Factors

The treatment effects of XENICAL were examined for 2 years in four of the five 1-year weight management clinical studies previously discussed (see Table 1). At the end of year 1, the patients' diets were reviewed and changed where necessary. The diet prescribed in the second year was designed to maintain patient's current weight. XENICAL was shown to be more effective than placebo in long-term weight control in four large, multicenter, 2-year double-blind, placebo-controlled studies.

Pooled data from four clinical studies indicate that 40% of all patients treated with 120 mg three times a day of XENICAL and 24% of patients treated with placebo who completed 2 years of the same therapy had ≥5% loss of body weight from randomization. Pooled data from four clinical studies indicate that the relative weight loss advantage between XENICAL 120 mg three times a day and placebo treatment groups was the same after 2 years as for 1 year, indicating that the pharmacologic advantage of XENICAL was maintained over 2 years. In the same studies cited in the **One-year Results** (see Table 1), the percentages of patients achieving a ≥5% and ≥10% weight loss after 2 years are shown in Table 3.

Table 3 Percentage of Patients Losing ≥5% and ≥10% of Body Weight From Randomization After 2-Year Treatment*

Intent-to-Treat Population†							
	≥5% Weight Loss				≥10% Weight Loss		
Study No.	XENICAL n	Placebo n	p-value	XENICAL n	Placebo n	p-value	
14119C	45.1% 133	23.6% 123	<0.001	24.8% 133	6.5% 123	<0.001	
14149	43.3% 178	27.2% 158	0.002	18.0% 178	9.5% 158	0.025	
14161‡	25.0% 148	15.0% 113	0.049	16.9% 148	3.5% 113	0.001	
14185	34.0% 147	27.9% 122	0.279	17.7% 147	11.5% 122	0.154	

The diet utilized during year 2 was designed for weight maintenance and not weight loss.

* Treatment designates XENICAL 120 mg three times a day plus diet or placebo plus diet

† Last observation carried forward

‡ All studies, with the exception of 14161 were conducted at centers specializing in treating obesity or complications of obesity. Study 14161 was conducted with primary care physicians.

The relative changes in risk factors associated with obesity following 2 years of therapy were also assessed in the population as a whole and the population with abnormal risk factors at randomization.

Population as a Whole

The relative differences in risk factors between treatment with XENICAL and placebo were similar to the results following 1 year of therapy for total cholesterol, LDL-cholesterol, LDL/HDL ratio, triglycerides, fasting glucose, fasting insulin, diastolic blood pressure, waist circumference, and hip circumference. The relative differences between treatment groups for HDL cholesterol and systolic blood pressure were less than that observed in the year one results.

Population With Abnormal Risk Factors at Randomization

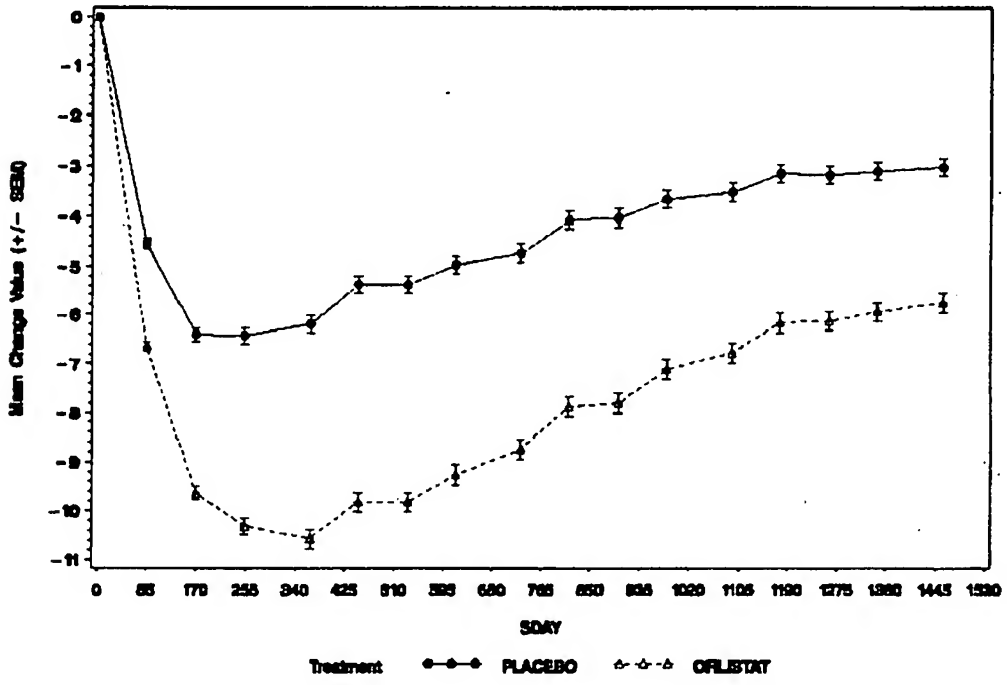
The relative differences in risk factors between treatment with XENICAL and placebo were similar to the results following 1 year of therapy for LDL- and HDL-cholesterol, triglycerides, fasting insulin, diastolic blood pressure, and waist circumference. The relative differences between treatment groups for LDL/HDL ratio and isolated systolic blood pressure were less than that observed in the year one results.

Four-Year Results: Long-term Weight Control and Risk Factors

In the 4-year double-blind, placebo-controlled XENDOS study, the effects of orlistat in delaying the onset of type 2 diabetes and on body weight were compared to placebo in 3304 obese patients who had either normal or impaired glucose tolerance at baseline. Thirty-four percent of the 1655 patients who were randomized to the placebo group and 52% of the 1649 patients who were randomized to the orlistat group completed the 4-year study.

At the end of the study, the mean percent weight loss in the placebo group was -2.75% compared with -5.17% in the orlistat group ($p<0.001$) (see Figure 1). Forty-five percent of the placebo patients and 73% of the orlistat patients lost ≥5% of their baseline body weight, and 21% of the placebo patients and 41% of the orlistat patients lost ≥10% of their baseline body weight following the first year of treatment. Following 4 years of treatment, 28% of the placebo patients and 45% of the orlistat patients lost ≥5% of their baseline body weight and 10% of the placebo patients and 21% of the orlistat patients lost ≥10% of their baseline body weight.

Figure 1 Mean Change from Baseline Body Weight (Kgs) Over Time



The relative changes from baseline in risk factors associated with obesity following 4 years of therapy were assessed in the XENDOS study population (see Table 4).

Table 4 Mean Change in Risk Factors From Randomization Following 4-Years Treatment*

Risk Factor	XENICAL 120 mg†	Placebo†
Metabolic:		
Total Cholesterol	-7.02%	-2.03%
LDL-Cholesterol	-11.66%	-3.85%
HDL-Cholesterol	+5.92%	+7.01%
LDL/HDL	-0.53	-0.33
Triglycerides	+3.64%	+1.30
Fasting Glucose, mmol/L	+0.12	+0.23
Fasting Insulin, pmol/L	-24.93	-15.71
Cardiovascular:		
Systolic Blood Pressure, mm Hg	-4.12	-2.60
Diastolic Blood Pressure, mm Hg	-1.93	-0.87
Anthropometric:		
Waist Circumference, cm	-5.78	-3.99

*Treatment designates XENICAL 120 mg three times a day plus diet or placebo plus diet
†Intent-to-treat population

Study of Patients With Type 2 Diabetes

A 1-year double-blind, placebo-controlled study in type 2 diabetics (N=321) stabilized on sulfonylureas was conducted. Thirty percent of patients treated with XENICAL achieved at least a 5% or greater reduction in body weight from randomization compared to 13% of the placebo-treated patients ($p<0.001$). Table 5 describes the changes over 1 year of treatment with XENICAL compared to placebo, in sulfonylurea usage and dose reduction as well as in hemoglobin HbA1c, fasting glucose, and insulin.

Table 5 Mean Changes in Body Weight and Glycemic Control From Randomization Following 1-Year Treatment in Patients With Type 2 Diabetes

	XENICAL 120 mg* (n=162)	Placebo* (n=159)	Statistical Significance
% patients who discontinued dose of oral sulfonylurea	11.7%	7.5%	†
% patients who decreased dose of oral sulfonylurea	31.5%	21.4%	
Average reduction in sulfonylurea medication dose	-22.8%	-9.1%	†
Body weight change (lbs)	-8.9	-4.2	†
HbA1c	-0.18%	+0.28%	†
Fasting glucose, mmol/L	-0.02	+0.54	†
Fasting insulin, pmol/L	-19.68	-18.02	ns

Statistical significance based on intent-to-treat population, last observation carried forward.

* Treatment designates XENICAL 120 mg three times a day plus diet or placebo plus diet

† Statistically significant ($p \leq 0.05$) based on intent-to-treat, last observation carried forward

ns nonsignificant, $p>0.05$

In addition, XENICAL (n=162) compared to placebo (n=159) was associated with significant lowering for total cholesterol (-1.0% vs +9.0%, p≤0.05), LDL-cholesterol (-3.0% vs +10.0%, p≤0.05), LDL/HDL ratio (-0.26 vs -0.02, p≤0.05) and triglycerides (+2.54% vs +16.2%, p≤0.05), respectively. For HDL cholesterol, there was a +6.49% increase on XENICAL and +8.6% increase on placebo, p>0.05. Systolic blood pressure increased by +0.61 mm Hg on XENICAL and increased by +4.33 mm Hg on placebo, p>0.05. Diastolic blood pressure decreased by -0.47 mm Hg for XENICAL and by -0.5 mm Hg for placebo, p>0.05.

Glucose Tolerance in Obese Patients

Two-year studies that included oral glucose tolerance tests were conducted in obese patients not previously diagnosed or treated for type 2 diabetes and whose baseline oral glucose tolerance test (OGTT) status at randomization was either normal, impaired, or diabetic.

The progression from a normal OGTT at randomization to a diabetic or impaired OGTT following 2 years of treatment with XENICAL (n=251) or placebo (n=207) were compared. Following treatment with XENICAL, 0.0% and 7.2% of the patients progressed from normal to diabetic and normal to impaired, respectively, compared to 1.9% and 12.6% of the placebo treatment group, respectively.

In patients found to have an impaired OGTT at randomization, the percent of patients improving to normal or deteriorating to diabetic status following 1 and 2 years of treatment with XENICAL compared to placebo are presented. After 1 year of treatment, 45.8% of the placebo patients and 73% of the XENICAL patients had a normal oral glucose tolerance test while 10.4% of the placebo patients and 2.6% of the XENICAL patients became diabetic. After 2 years of treatment, 50% of the placebo patients and 71.7% of the XENICAL patients had a normal oral glucose tolerance test while 7.5% of placebo patients were found to be diabetic and 1.7% of XENICAL patients were found to be diabetic after treatment.

Onset of Type 2 Diabetes In Obese Patients

In the XENDOS trial, in the overall population, orlistat delayed the onset of type 2 diabetes such that at the end of four years of treatment the cumulative incidence rate of diabetes was 8.3% for the placebo group compared to 5.5% for the orlistat group, p=0.01 (see Table 6). This finding was driven by a statistically-significant reduction in the incidence of developing type 2 diabetes in those patients who had impaired glucose tolerance at baseline (Table 6 and Figure 2). Orlistat did not reduce the risk for the development of diabetes in patients with normal glucose tolerance at baseline.

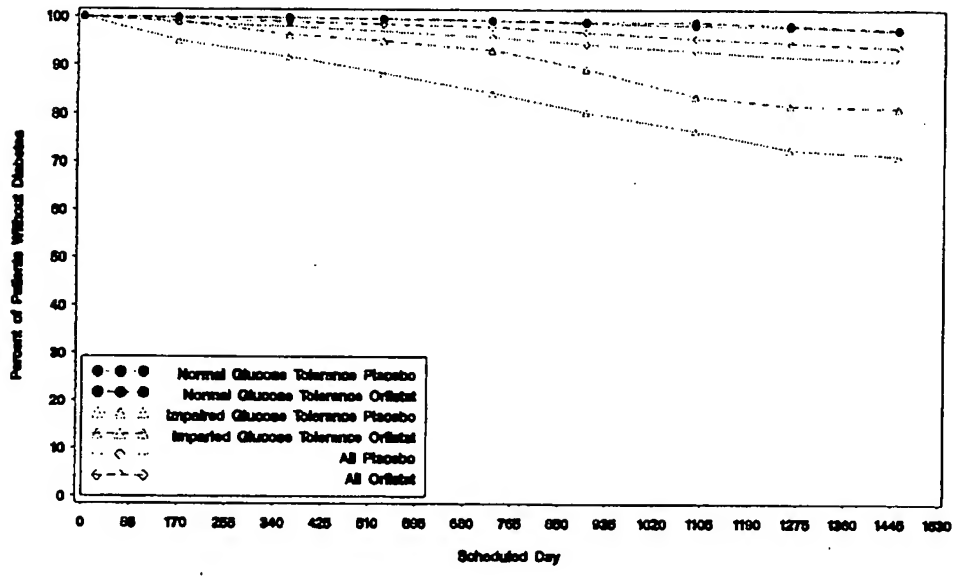
The effect of XENICAL to delay the onset of type 2 diabetes in obese patients with IGT is presumably due to weight loss, and not to any independent effects of the drug on glucose or insulin metabolism. The effect of orlistat on weight loss is adjunctive to diet and exercise.

Table 6 Incidence Rate of Diabetes at Year 4 by OGTT Status at Baseline*

OGTT at baseline	Normal		Impaired		All	
Treatment	Placebo	Orlistat	Placebo	Orlistat	Placebo	Orlistat
Number of patients*	1148	1235	324	337	1472	1572
# pts developing diabetes	16	21	62	48	78	69
Life table rate†	2.1%	1.7%	27.2%	18.7%	8.3%	5.5%
Observed percent	1.4%	1.7%	19.1%	14.2%	5.3%	4.4%
Absolute risk reduction						
Life table	0.4%		8.5%		2.8%	
Observed	-0.3%		4.9%		0.9%	
Relative risk reduction††	8%		42%		34%	
p-value	0.79		<0.01		0.01	

*Based on patients with a baseline and at least one follow-up OGTT measurement
†Rate adjusted for dropouts
†† Computed as (1- hazard ratio)

Figure 2 Percentage of Patients Without Diabetes Over Time



Pediatric Clinical Studies

The effects of XENICAL on body mass index (BMI) and weight loss were assessed in a 54-week multicenter, double-blind, placebo-controlled study in 539 obese adolescents (357 receiving XENICAL 120 mg three times a day, 182 receiving placebo), aged 12 to 16 years. All study participants had a baseline BMI that was 2 units greater than the US weighted mean for the 95th percentile based on age and gender. Body mass index was the primary efficacy parameter because it takes into account changes in height and body weight, which occur in growing children.

During the study, all patients were instructed to take a multivitamin containing fat-soluble vitamins at least 2 hours before or after ingestion of XENICAL. Patients were also maintained on a well-balanced, reduced-calorie diet that was intended to provide 30% of calories from fat. In addition, all patients were placed on a behavior modification program and offered exercise counseling.

Approximately 65% of patients in each treatment group completed the study.

Following one year of treatment, BMI decreased by an average of 0.55 kg/m^2 in the XENICAL-treated patients and increased by an average of 0.31 kg/m^2 in the placebo-treated patients (p=0.001).

The percentages of patients achieving ≥5% and ≥10% reduction in BMI and body weight after 52 weeks of treatment for the intent-to-treat population are presented in Table 7.

Table 7 **Percentages of Patients with ≥5% and ≥10% Decrease in Body Mass Index and Body Weight After 1-Year Treatment* (Protocol NM16189)**

	Intent-to-Treat Population†			
	≥5% Decrease		≥10% Decrease	
	XENICAL n	Placebo n	XENICAL n	Placebo n
BMI	26.5% 347	15.7% 178	13.3% 347	4.5% 178
Body Weight	19.0% 348	11.7% 180	9.5% 348	3.3% 180

* Treatment designates XENICAL 120 mg three times a day plus diet or placebo plus diet

† Last observation carried forward

INDICATIONS AND USAGE

XENICAL is indicated for obesity management including weight loss and weight maintenance when used in conjunction with a reduced-calorie diet. XENICAL is also indicated to reduce the risk for weight regain after prior weight loss. XENICAL is indicated for obese patients with an initial body mass index (BMI) ≥30 kg/m^2 or ≥27 kg/m^2 in the presence of other risk factors (eg, hypertension, diabetes, dyslipidemia).

Table 8 illustrates body mass index (BMI) according to a variety of weights and heights. The BMI is calculated by dividing weight in kilograms by height in meters squared. For example, a person who weighs 180 lbs and is 5'5" would have a BMI of 30.

Table 8 **Body Mass Index (BMI), kg/m^2***

HEIGHT (ft/in)	WEIGHT (lb)																				
	120	130	140	150	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300	310	320
4'10"	25	27	29																		
4'11"	24	26	28																		
5'0"	23	25	27	29																	
5'1"	23	25	27	28																	
5'2"	22	24	26	27	29																
5'3"	21	23	25	27	28																
5'4"	21	22	24	26	28	29															
5'5"	20	22	23	25	27	28															
5'6"	19	21	23	24	26	27	29														
5'7"	19	20	22	24	25	27	28														
5'8"	18	20	21	23	24	26	27	29													
5'9"	18	19	21	22	24	25	27	28													
5'10"	17	19	20	22	23	24	26	27	29												
5'11"	17	18	20	21	22	24	25	27	28	29											
6'0"	16	18	19	20	22	23	24	26	27	29											
6'1"	16	17	19	20	21	22	24	25	26	28	29										
6'2"	15	17	18	19	21	22	23	24	26	27	28										

* Conversion Factors:
Weight in lbs ÷ 2.2 = weight in kilograms (kg)
Height in inches × 0.0254 = height in meters (m)
1 foot = 12 inches

CONTRAINDICATIONS

XENICAL is contraindicated in patients with chronic malabsorption syndrome or cholestasis, and in patients with known hypersensitivity to XENICAL or to any component of this product.

WARNINGS

Miscellaneous

Organic causes of obesity (eg, hypothyroidism) should be excluded before prescribing XENICAL.

Preliminary data from a XENICAL and cyclosporine drug interaction study indicate a reduction in cyclosporine plasma levels when XENICAL was coadministered with cyclosporine. Therefore, XENICAL and cyclosporine should not be coadministered. To reduce the chance of a drug-drug interaction, cyclosporine should be taken at least 2 hours before or after XENICAL in patients taking both drugs. In addition, in those patients whose cyclosporine levels are being measured, more frequent monitoring should be considered.

PRECAUTIONS

General

Patients should be advised to adhere to dietary guidelines (see DOSAGE AND ADMINISTRATION). Gastrointestinal events (see ADVERSE REACTIONS) may increase when XENICAL is taken with a diet high in fat (>30% total daily calories from fat). The daily intake of fat should be distributed over three main meals. If XENICAL is taken with any one meal very high in fat, the possibility of gastrointestinal effects increases.

Patients should be strongly encouraged to take a multivitamin supplement that contains fat-soluble vitamins to ensure adequate nutrition because XENICAL has been shown to reduce the absorption of some fat-soluble vitamins and beta-carotene (see DOSAGE AND ADMINISTRATION). In addition, the levels of vitamin D and beta-carotene may be low in obese patients compared with non-obese subjects. The supplement should be taken once a day at least 2 hours before or after the administration of XENICAL, such as at bedtime.

Table 9 illustrates the percentage of adult patients on XENICAL and placebo who developed a low vitamin level on two or more consecutive visits during 1 and 2 years of therapy in studies in which patients were not previously receiving vitamin supplementation.

Table 9 **Incidence of Low Vitamin Values on Two or More Consecutive Visits (Nonsupplemented Adult Patients With Normal Baseline Values - First and Second Year)**

	Placebo*	XENICAL*
Vitamin A	1.0%	2.2%
Vitamin D	6.6%	12.0%
Vitamin E	1.0%	5.8%
Beta-carotene	1.7%	6.1%

* Treatment designates placebo plus diet or XENICAL plus diet

Table 10 illustrates the percentage of adolescent patients on XENICAL and placebo who developed a low vitamin level on two or more consecutive visits during the 1-year study.

Table 10 **Incidence of Low Vitamin Values on Two or More Consecutive Visits (Pediatric Patients With Normal Baseline Values*)**

	Placebo†	XENICAL†
Vitamin A	0.0%	0.0%
Vitamin D	0.7%	1.4%
Vitamin E	0.0%	0.0%
Beta-carotene	0.8%	1.5%

* All patients were treated with vitamin supplementation throughout the course of the study

† Treatment designates placebo plus diet or XENICAL plus diet

Some patients may develop increased levels of urinary oxalate following treatment with XENICAL. Caution should be exercised when prescribing XENICAL to patients with a history of hyperoxaluria or calcium oxalate nephrolithiasis.

Weight-loss induction by XENICAL may be accompanied by improved metabolic control in diabetics, which might require a reduction in dose of oral hypoglycemic medication (eg, sulfonylureas, metformin) or insulin (see CLINICAL STUDIES).

Misuse Potential

As with any weight-loss agent, the potential exists for misuse of XENICAL in inappropriate patient populations (eg, patients with anorexia nervosa or bulimia). See INDICATIONS AND USAGE for recommended prescribing guidelines.

Information for Patients

Patients should read the Patient Information before starting treatment with XENICAL and each time their prescription is renewed.

Drug Interactions

Alcohol

In a multiple-dose study in 30 normal-weight subjects, coadministration of XENICAL and 40 grams of alcohol (eg, approximately 3 glasses of wine) did not result in alteration of alcohol pharmacokinetics, orlistat pharmacodynamics (fecal fat excretion), or systemic exposure to orlistat.

Cyclosporine

Preliminary data from a XENICAL and cyclosporine drug interaction study indicate a reduction in cyclosporine plasma levels when XENICAL was coadministered with cyclosporine (see WARNINGS).

Digoxin

In 12 normal-weight subjects receiving XENICAL 120 mg three times a day for 6 days, XENICAL did not alter the pharmacokinetics of a single dose of digoxin.

Fat-soluble Vitamin Supplements and Analogues

A pharmacokinetic interaction study showed a 30% reduction in beta-carotene supplement absorption when concomitantly administered with XENICAL. XENICAL inhibited absorption of a vitamin E acetate supplement by approximately 60%. The effect of orlistat on the absorption of supplemental vitamin D, vitamin A, and nutritionally-derived vitamin K is not known at this time.

Glyburide

In 12 normal-weight subjects receiving orlistat 80 mg three times a day for 5 days, orlistat did not alter the pharmacokinetics or pharmacodynamics (blood glucose-lowering) of glyburide.

Nifedipine (extended-release tablets)

In 17 normal-weight subjects receiving XENICAL 120 mg three times a day for 6 days, XENICAL did not alter the bioavailability of nifedipine (extended-release tablets).

Oral Contraceptives

In 20 normal-weight female subjects, the treatment of XENICAL 120 mg three times a day for 23 days resulted in no changes in the ovulation-suppressing action of oral contraceptives.

Phenytoin

In 12 normal-weight subjects receiving XENICAL 120 mg three times a day for 7 days, XENICAL did not alter the pharmacokinetics of a single 300-mg dose of phenytoin.

Pravastatin

In a 2-way crossover study of 24 normal-weight, mildly hypercholesterolemic patients receiving XENICAL 120 mg three times a day for 6 days, XENICAL did not affect the pharmacokinetics of pravastatin.

Warfarin

In 12 normal-weight subjects, administration of XENICAL 120 mg three times a day for 16 days did not result in any change in either warfarin pharmacokinetics (both R- and S-enantiomers) or pharmacodynamics (prothrombin time and serum Factor VII). Although undercarboxylated osteocalcin, a marker of vitamin K nutritional status, was unaltered with XENICAL administration, vitamin K levels tended to decline in subjects taking XENICAL. Therefore, as vitamin K absorption may be decreased with XENICAL, patients on chronic stable doses of warfarin who are prescribed XENICAL should be monitored closely for changes in coagulation parameters.

Carcinogenesis, Mutagenesis, Impairment of Fertility

Carcinogenicity studies in rats and mice did not show a carcinogenic potential for orlistat at doses up to 1000 mg/kg/day and 1500 mg/kg/day, respectively. For mice and rats, these doses are 38 and 46 times the daily human dose calculated on an area under concentration vs time curve basis of total drug-related material.

Orlistat had no detectable mutagenic or genotoxic activity as determined by the Ames test, a mammalian forward mutation assay (V79/HPRT), an in vitro clastogenesis assay in peripheral human lymphocytes, an unscheduled DNA synthesis assay (UDS) in rat hepatocytes in culture, and an in vivo mouse micronucleus test.

When given to rats at a dose of 400 mg/kg/day in a fertility and reproduction study, orlistat had no observable adverse effects. This dose is 12 times the daily human dose calculated on a body surface area (mg/m^2) basis.

Pregnancy

Teratogenic Effects: Pregnancy Category B.

Teratogenicity studies were conducted in rats and rabbits at doses up to 800 mg/kg/day. Neither study showed embryotoxicity or teratogenicity. This dose is 23 and 47 times the daily human dose calculated on a body surface area (mg/m^2) basis for rats and rabbits, respectively.

The incidence of dilated cerebral ventricles was increased in the mid- and high-dose groups of the rat teratology study. These doses were 6 and 23 times the daily human dose calculated on a body surface area (mg/m^2) basis for the mid- and high-dose levels, respectively. This finding was not reproduced in two additional rat teratology studies at similar doses.

There are no adequate and well-controlled studies of XENICAL in pregnant women. Because animal reproductive studies are not always predictive of human response, XENICAL is not recommended for use during pregnancy.

Nursing Mothers

It is not known if orlistat is secreted in human milk. Therefore, XENICAL should not be taken by nursing women.

Pediatric Use

The safety and efficacy of XENICAL have been evaluated in obese adolescent patients aged 12 to 16 years. Use of XENICAL in this age group is supported by evidence from adequate and well-controlled studies of XENICAL in adults with additional data from a 54-week efficacy and safety study and a 21-day mineral balance study in obese adolescent patients aged 12 to 16 years. Patients treated with XENICAL had a mean reduction in BMI of 0.55 kg/m^2 compared with an average increase of 0.31 kg/m^2 in placebo-treated patients (p=0.001). In both adolescent studies, adverse effects were generally similar to those described in adults and included fatty/oily stool, oily spotting, and oily evacuation. In a subgroup of 152 orlistat and 77 placebo patients from the 54-week study, changes in body composition measured by DEXA were similar in both treatment groups with the exception of fat mass, which was significantly reduced in patients treated with XENICAL compared to patients treated with placebo (-2.5 kg vs -0.6 kg, p=0.033). Because XENICAL can interfere with the absorption of fat-soluble vitamins, all patients should take a daily multivitamin that contains vitamins A, D, E, K, and beta-carotene. The supplement should be taken at least 2 hours before or after XENICAL (see CLINICAL PHARMACOLOGY: Other Short-term Studies; CLINICAL STUDIES: Pediatric Clinical Studies; ADVERSE REACTIONS: Pediatric Patients). XENICAL has not been studied in pediatric patients below the age of 12 years.

Geriatric Use

Clinical studies of XENICAL did not include sufficient numbers of patients aged 65 years and older to determine whether they respond differently from younger patients.

ADVERSE REACTIONS

Commonly Observed (based on first year and second year data - XENICAL 120 mg three times a day versus placebo):

Gastrointestinal (GI) symptoms were the most commonly observed treatment-emergent adverse events associated with the use of XENICAL in the seven double-blind, placebo-controlled clinical trials and are primarily a manifestation of the mechanism of action. (Commonly observed is defined as an incidence of ≥5% and an incidence in the XENICAL 120 mg group that is at least twice that of placebo.)

Table 11 Commonly Observed Adverse Events

Adverse Event	Year 1		Year 2	
	XENICAL* % Patients (N=1913)	Placebo* % Patients (N=1466)	XENICAL* % Patients (N=613)	Placebo* % Patients (N=524)
Oily Spotting	26.6	1.3	4.4	0.2
Flatus with Discharge	23.9	1.4	2.1	0.2
Fecal Urgency	22.1	6.7	2.8	1.7
Fatty/Oily Stool	20.0	2.9	5.5	0.6
Oily Evacuation	11.9	0.8	2.3	0.2
Increased Defecation	10.8	4.1	2.6	0.8
Fecal Incontinence	7.7	0.9	1.8	0.2

* Treatment designates XENICAL three times a day plus diet or placebo plus diet

These and other commonly observed adverse reactions were generally mild and transient, and they decreased during the second year of treatment. In general, the first occurrence of these events was within 3 months of starting therapy. Overall, approximately 50% of all episodes of GI adverse events associated with orlistat treatment lasted for less than 1 week, and a majority lasted for no more than 4 weeks. However, GI adverse events may occur in some individuals over a period of 6 months or longer.

Discontinuation of Treatment

In controlled clinical trials, 8.8% of patients treated with XENICAL discontinued treatment due to adverse events, compared with 5.0% of placebo-treated patients. For XENICAL, the most common adverse events resulting in discontinuation of treatment were gastrointestinal.

Incidence in Controlled Clinical Trials

The following table lists other treatment-emergent adverse events from seven multicenter, double-blind, placebo-controlled clinical trials that occurred at a frequency of ≥2% among patients treated with XENICAL 120 mg three times a day and with an incidence that was greater than placebo during year 1 and year 2, regardless of relationship to study medication.

Table 12 Other Treatment-Emergent Adverse Events From Seven Placebo-Controlled Clinical Trials

Body System/Adverse Event	Year 1		Year 2	
	XENICAL* % Patients (N=1913)	Placebo* % Patients (N=1466)	XENICAL* % Patients (N=613)	Placebo* % Patients (N=524)
Gastrointestinal System				
Abdominal Pain/Discomfort	25.5	21.4	–	–
Nausea	8.1	7.3	3.6	2.7
Infectious Diarrhea	5.3	4.4	–	–
Rectal Pain/Discomfort	5.2	4.0	3.3	1.9
Tooth Disorder	4.3	3.1	2.9	2.3
Gingival Disorder	4.1	2.9	2.0	1.5
Vomiting	3.8	3.5	–	–
Respiratory System				
Influenza	39.7	36.2	–	–
Upper Respiratory Infection	38.1	32.8	26.1	25.8
Lower Respiratory Infection	7.8	6.6	–	–
Ear, Nose & Throat Symptoms	2.0	1.6	–	–
Musculoskeletal System				
Back Pain	13.9	12.1	–	–
Pain Lower Extremities	–	–	10.8	10.3
Arthritis	5.4	4.8	–	–
Myalgia	4.2	3.3	–	–
Joint Disorder	2.3	2.2	–	–
Tendonitis	–	–	2.0	1.9
Central Nervous System				
Headache	30.6	27.6	–	–
Dizziness	5.2	5.0	–	–
Body as a Whole				
Fatigue	7.2	6.4	3.1	1.7
Sleep Disorder	3.9	3.3	–	–
Skin & Appendages				
Rash	4.3	4.0	–	–
Dry Skin	2.1	1.4	–	–
Reproductive, Female				
Menstrual Irregularity	9.8	7.5	–	–
Vaginitis	3.8	3.6	2.6	1.9
Urinary System				
Urinary Tract Infection	7.5	7.3	5.9	4.8
Psychiatric Disorder				
Psychiatric Anxiety	4.7	2.9	2.8	2.1
Depression	–	–	3.4	2.5
Hearing & Vestibular Disorders				
Otitis	4.3	3.4	2.9	2.5
Cardiovascular Disorders				
Pedal Edema	–	–	2.8	1.9

* Treatment designates XENICAL 120 mg three times a day plus diet or placebo plus diet

– None reported at a frequency ≥2% and greater than placebo

In the 4-year XENDOS study, the general pattern of adverse events was similar to that reported for the 1- and 2-year studies with the total incidence of gastrointestinal-related adverse events occurring in year 1 decreasing each year over the 4-year period.

Other Clinical Studies or Postmarketing Surveillance

Rare cases of hypersensitivity have been reported with the use of XENICAL. Signs and symptoms have included pruritus, rash, urticaria, angioedema, bronchospasm and anaphylaxis. Very rare cases of bullous eruption, increase in transaminases and in alkaline phosphatase, and exceptional cases of hepatitis that may be serious have been reported. No causal relationship or physiopathological mechanism between hepatitis and orlistat therapy has been established. Reports of decreased prothrombin, increased INR and unbalanced anticoagulant treatment resulting in change of hemostatic parameters have been reported in patients treated concomitantly with orlistat and anticoagulants.

In clinical trials in obese diabetic patients, hypoglycemia and abdominal distension were also observed.

Preliminary data from a XENICAL and cyclosporine drug interaction study indicate a reduction in cyclosporine plasma levels when XENICAL was coadministered with cyclosporine (see WARNINGS).

Pediatric Patients

In clinical trials with XENICAL in adolescent patients ages 12 to 16 years, the profile of adverse reactions was generally similar to that observed in adults.

OVERDOSAGE

Single doses of 800 mg XENICAL and multiple doses of up to 400 mg three times a day for 15 days have been studied in normal weight and obese subjects without significant adverse findings.

Should a significant overdose of XENICAL occur, it is recommended that the patient be observed for 24 hours. Based on human and animal studies, systemic effects attributable to the lipase-inhibiting properties of orlistat should be rapidly reversible.

DOSAGE AND ADMINISTRATION

The recommended dose of XENICAL is one 120-mg capsule three times a day with each main meal containing fat (during or up to 1 hour after the meal).

The patient should be on a nutritionally balanced, reduced-calorie diet that contains approximately 30% of calories from fat. The daily intake of fat, carbohydrate, and protein should be distributed over three main meals. If a meal is occasionally missed or contains no fat, the dose of XENICAL can be omitted.

Because XENICAL has been shown to reduce the absorption of some fat-soluble vitamins and beta-carotene, patients should be counseled to take a multivitamin containing fat-soluble vitamins to ensure adequate nutrition (see PRECAUTIONS:

General). The supplement should be taken at least 2 hours before or after the administration of XENICAL, such as at bedtime.

Doses above 120 mg three times a day have not been shown to provide additional benefit.

Based on fecal fat measurements, the effect of XENICAL is seen as soon as 24 to 48 hours after dosing. Upon discontinuation of therapy, fecal fat content usually returns to pretreatment levels within 48 to 72 hours.

The safety and effectiveness of XENICAL beyond 4 years have not been determined at this time.

HOW SUPPLIED

XENICAL is a dark-blue, hard-gelatin capsule containing pellets of powder.

XENICAL 120 mg Capsules: Dark-blue, two-piece, No. 1 opaque hard-gelatin capsule imprinted with Roche and XENICAL 120 in light-blue ink — bottle of 90 (NDC 0004-0256-52).

Storage Conditions

Store at 25°C (77°F); excursions permitted to 15° to 30°C (59° to 86°F) [see USP Controlled Room Temperature]. Keep bottle tightly closed.

XENICAL should not be used after the given expiration date.

Distributed by:

Pharmaceuticals

Roche Laboratories Inc.
340 Kingsland Street
Nutley, New Jersey 07110-1199

27898887

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HYPOGLYCEMIC ACTIVITY OF SEVERAL SEAWEED EXTRACTS

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Summary

The hypoglycemic activity of several seaweed extracts on rabbits was studied. Ethanol extracts of *Laminaria ochroleuca*, *Saccorhiza polyschides* and *Fucus vesiculosus* were administered orally to normal animals and their effects on glycemia and triglyceridemia evaluated. Crude polysaccharides and protein solutions from *Himanthalia elongata* and *Codium tomentosum* were also assayed. Polysaccharides and proteins from *H. elongata* caused a significant reduction in blood glucose 8 h after intravenous administration. A dose of 5 mg/kg of crude polysaccharide lowered glycemia about 18% in normal rabbits and by about 50% in alloxan-diabetic animals, while the protein solution lowered glycemia in diabetic rabbits by about 30%.

Introduction

Several seaweeds have been reported to include hypoglycemic capacity among their biological activities. Certain *Sargassum*, *Cystoseira* (Phaeophyceae), *Corallina* and *Pterocladia* (Rhodophyceae) species have been shown to lower blood glucose and serum lipid levels (Bézanger-Beauquesne, 1982). *Laminaria* species which are employed against goitre in folk medicine also lower blood pressure and cholesterol levels (Hoppe, 1979), and *Fucus* has been used against goitre, obesity and diabetes (Lyon de Castro, 1981). Several algal macromolecule, polysaccharide and protein preparations have also been reported to be hypocholesterolemics and hypoglycemiants (Güven et al., 1979).

In this work, ethanolic extracts and crude polysaccharide and protein extracts from various seaweeds were assayed for their hypoglycemic properties in rabbits. The effects of the ethanol extracts on serum triglycerides were also studied.

Materials and methods

Seaweed collection and handling

The following marine algae were studied: *Laminaria ochroleuca* De la Pylaie (Laminariaceae), *Saccorhiza polyschides* (Lightfoot) Batters (Laminariaceae), *Fucus vesiculosus* L. (Fucaceae), *Himanthalia elongata* (L.) S.F. Gray (Himanthaliaceae), *Codium tomentosum* (Huds.) Stackhouse (Codiaceae).

In July–August 1986 and September 1987 *L. ochroleuca*, *F. vesiculosus*, *H. elongata* and *C. tomentosum* were hand picked at low tide on Porto Nadelas beach (La Coruña, Spain) and *S. polyschides* fronds were obtained in the Ria de Arosa area (Pontevedra, Spain). All specimens were authenticated by the Plant Biology Department of the University of Santiago de Compostela, Spain.

The fresh algal fronds were washed with tap water and cut in pieces. Part of this material was dried in a forced-air oven at 40 °C and stored.

Preparation of extracts

Ethanolic extracts. Fresh algae were stabilized with boiling 95% ethanol for 1 h. The resulting extracts (extract 1) were concentrated to dryness in vacuo at 40 °C, and the stabilized algae pieces were dried, powdered, stored and later extracted again with 95% ethanol to yield extract 2. Extract 2 was concentrated to a small volume in vacuo and added to the dry extract 1, and the resulting solution was evaporated to dryness in vacuo, dissolved in distilled water and stored at −20 °C until use. Percentage yields were as follows: *L. ochroleuca*, 21.2%; *S. polyschides*, 34.6%; *F. vesiculosus*, 24.1%.

Water-extractable polysaccharides. Dried seaweed samples (100g) were extracted for 4 h with 4 l of deionized water in a boiling water bath, with occasional stirring. The extracts were then filtered with suction through filter paper and 4 volumes of 95% ethanol were stirred into the filtrate at alkaline pH to precipitate polysaccharides (Su and Hassid, 1962). A 49.5% yield of polysaccharide was obtained from *H. elongata* and 2.2% from *C. tomentosum*. The sugar contents, as determined by the phenol-H_2SO_4 method (Dubois et al., 1956) and estimated as glucose, were 23% for *H. elongata* and 15% for *C. tomentosum*. Samples of polysaccharides were dissolved in deionized water and dialyzed against water in cellulose tubing (Sigma) for 48 h. The non-dialyzed portions were used to assay for hypoglycemic activity.

Protein extraction. Algal protein was isolated by the method of Güven and Güler (1979). Fresh algae were macerated with 2% sodium carbonate solution for 24 h. The extract was filtered and acidified and 3% barium chloride was added to the solution. The precipitate obtained was separated and extracted with 1% sodium carbonate and then filtered. The filtrate was neutralized and dialyzed as above. The non-dialyzed portion was used. The protein content was determined by the method of Lowry et al. (1951). Percentage yields by this technique were: *H. elongata*, 0.83%; *C. tomentosum*, 0.02%.

Biological assays

The animals used were normal male New Zealand rabbits weighing approximately 2 kg and fed mixed feeds (Saprogal) with free access to tap water. All assays were performed on animals fasted for 20 h but with water allowed ad libitum. All doses are expressed as g or mg equivalents of dried seaweed per kg of body weight, except for polysaccharides, which are expressed as mg of polysaccharide extract per kg of body weight. Ethanol extracts were administered intragastrically (10 ml/kg). Polysaccharide and protein extracts were administered intravenously (1 ml/kg) via the ear vein. All blood samples were withdrawn by puncture from the contralateral ear vein.

Effect on normoglycemic animals

Doses of 5, 10 and 20 g/kg of ethanol extracts from *L. ochroleuca*, *S. polyschides* and *F. vesiculosus* were administered to the fasted rabbits. Blood samples were taken immediately before administration (0 h) and +2, +4 and +6 h later. The doses assayed for *H. elongata* were 2.5, 5 and 10 mg/kg for polysaccharides and 100, 200 and 400 mg/kg of protein solution and for *C. tomentosum*, 5 and 10 mg/kg for polysaccharides and 500 and 900 mg/kg for protein solution. When polysaccharide and protein solutions were administered, blood samples were taken at 0, +1, +3, +6 and +8 h. The concentration of glucose in serum samples was measured in a Seralyzer reflectance photometer (Ames, Miles Laboratories) using Seralyzer reagent strips. Percentage variations of glycemia with respect to the initial (0 h) level was calculated (Lamela et al., 1985). Animals dosed with distilled water were used as controls.

Alloxan-diabetic rabbits

Chronically hyperglycemic rabbits were obtained by intravenous injection (in the ear vein) of 150 mg/kg of alloxan monohydrate (Sigma) dissolved in 0.9% saline (Akhtar and Ali, 1984). Seven days after administration, serum glucose levels of surviving rabbits (about 80%) were determined. Animals with fasting glycemia of 300 mg% or more were used. Polysaccharide and protein extracts from *H. elongata* were assayed. Doses of 5 mg/kg for polysaccharide and 200 mg/kg for protein solution were administered intravenously in the ear vein and blood samples were taken at 0, +1, +3, +6 and +8 h.

Effect of ethanol extracts on serum triglycerides

Ethanol extracts from *L. ochroleuca*, *S. polyschides* and *F. vesiculosus* were administered to normoglycemic animals as above, serum triglycerides were determined using a Seralyzer reflectance photometer (Ames, Miles Laboratories).

Statistical analysis

Data are given as means ± S.E. Differences between groups were

TABLE 1

EFFECT OF ETHANOLIC SEAWEED EXTRACTS ON SERUM GLUCOSE LEVELS OF NORMAL RABBITS

Treatment	Oral dosage (g/kg)	Initial glucose level (mg%)	Glycemia change (%)		
		0 h	+2 h	+4 h	+6 h
Control		125.5 ± 3.5	5.0 ± 1.7	3.5 ± 2.1	0.0 ± 2.0
L. ochroleuca	5	113.7 ± 4.6	5.2 ± 6.0	7.0 ± 5.9	6.7 ± 4.5
	10	115.8 ± 2.9	4.5 ± 3.9	−1.2 ± 3.2	4.2 ± 4.7
	20	128.0 ± 2.4	7.4 ± 4.7	−5.6 ± 4.1	−0.9 ± 2.2
S. polyschides	5	157.3 ± 5.5	−3.2 ± 2.6	−8.6 ± 4.9	−8.7 ± 5.6
	10	141.6 ± 5.1	−5.3 ± 4.7	−4.8 ± 4.4	−5.2 ± 3.9
	20	138.6 ± 6.4	1.1 ± 4.7	2.4 ± 6.3	2.2 ± 5.9
F. vesiculosus	5	120.5 ± 6.8	6.9 ± 8.7	14.1 ± 7.8	4.8 ± 7.8
	10	124.3 ± 2.1	−5.8 ± 1.6*	−9.6 ± 1.2*	3.0 ± 2.3
	20	127.5 ± 3.1	15.9 ± 6.2	6.1 ± 6.9	6.1 ± 2.2

Each value represents the mean ± S.E.M. of 6 rabbits.
*Significantly different from control, $P < 0.01$.

evaluated statistically using Student's *t* test (Tallarida and Murray, 1986). *P* values < 0.01 were taken to indicate significance.

Results and discussion

The yields of the ethanol extraction procedure were very high, especially the 34.6% of *S. polyschides*. This was probably due to the dilution of ethanol extractant during stabilization of the fresh seaweeds, which have a high water content, with the result that dissolved salts contribute to the final extract.

The low polysaccharide and protein contents of *C. tomentosum* (2.2% and 0.02% respectively) are also noteworthy. The purpose of dialyzing the polysaccharide and protein solutions was of course to remove salts and other molecules of low molecular weight.

Table 1 lists the effects of the ethanolic extracts of *L. ochroleuca*, *S. polyschides* and *F. vesiculosus* extracts on the glycemia of normal rabbits. Of all the assays performed, only oral administration of 10 g/kg of *F. vesicolosus* extract caused a statistically significant reduction of blood glucose, and even in this case the reduction was only slight (5.8% at +2 h and 9.6% at +4 h). Doubt is thrown on its true significance by the absence of any effect with 20 g/kg doses.

Table 2 lists the effects of the same ethanolic extracts on serum triglyceride levels. Oral administration of 20 g/kg of *L. ochroleuca* extract produced a statistically significant 20% reduction at +4 h, and triglyceride levels were still 18% below those of the controls at +6 h, although this difference was no longer statistically significant. *S. polyschides* extracts, on the other hand, increased serum triglyceride levels by a statistically significant 36% 6 h after administration of a 20 g/kg dose. *F. vesiculosus* had no effect at all on triglycerides at the dosages assayed.

Although the relationship between lipid and glucose metabolism suggest, a priori, that the glycemia and triglyceride results of Tables 1 and 2 ought to be related, they seem, in actual fact, to be quite independent of each other.

Table 3 shows that the results of assaying seaweed polysaccharides and protein solutions were more satisfactory, at least in the case of *H. elongata*. Intravenous administration of 5 mg/kg of crude *H. elongata* polysaccharides caused a significant 18% drop in the glycemia of normal animals at +8 h, and at 10 mg/kg a drop that was smaller (13.6%) but still significant. Analogous results were obtained by intravenous injection of crude *H. elongata* protein: doses of 200 and 400 mg/kg achieved respectively 17.7% and 18.2% reductions in glycemia at +8 h. In blood samples taken from randomly selected rabbits 24 h after injection of these dosages, glycemia had returned to its initial level (results not shown in Table 3). For some of the dosages assayed, smaller reductions in glycemia were already apparent sooner than 8 h after administration. Neither protein nor polysaccharide from *C. tomentosum* produced any significant change in glycemia by +8 h, though slight reductions were observed at +1 h after administration.

TABLE 2

EFFECT OF ETHANOLIC SEAWEED EXTRACTS ON SERUM TRIGLYCERIDES (TG)

Treatment	Oral dosage (g/kg)	Initial TG level (mg%)	TG change (%)	
		0 h	+4 h	+6 h
Control		61.0 ± 4.6	3.4 ± 4.7	−4.5 ± 5.5
L. ochroleuca	5	81.5 ± 4.7	5.2 ± 9.1	10.8 ± 7.7
	10	91.3 ± 3.0	−10.9 ± 2.7	−12.5 ± 7.1
	20	68.5 ± 4.6	−20.4 ± 2.3*	−18.4 ± 3.2
S. polyschides	5	97.0 ± 5.8	10.8 ± 8.3	8.3 ± 5.5
	20	52.7 ± 5.5	17.5 ± 7.0	31.7 ± 6.2*
F. vesiculosus	5	93.8 ± 12.0	0.0 ± 8.1	−3.0 ± 2.4
	10	59.2 ± 7.1	6.5 ± 2.6	6.3 ± 4.1
	20	57.2 ± 7.1	−0.9 ± 8.9	−7.7 ± 4.7

Values are mean ± S.E.M. (4–6 rabbits/group).
*Significantly different from control, $P < 0.01$.

TABLE 3

EFFECTS OF POLYSACCHARIDE AND PROTEIN EXTRACTS ON NORMOGLYCEMIC RABBITS

Treatment	Intravenous dosage (mg/kg)	Initial glucose level (mg%)	Glycemia change (%)			
		0 h	+1 h	+3 h	+6 h	+8 h
Control		140.2 ± 1.8	3.9 ± 1.8	−4.0 ± 1.8	−3.9 ± 1.5	−4.0 ± 1.9
Polysaccharides						
C. tomentosum	5	149.5 ± 1.4	−4.6 ± 2.0*	−5.9 ± 2.3	−7.8 ± 1.4	−8.4 ± 1.6
	10	138.3 ± 1.7	−2.2 ± 1.5	2.1 ± 1.5	−0.8 ± 2.5	1.2 ± 2.2
H. elongata	2.5	145.6 ± 3.2	−2.5 ± 2.5	5.2 ± 3.2	−12.5 ± 2.7	−9.6 ± 2.0
	5	139.4 ± 2.3	−6.6 ± 2.4*	−1.5 ± 1.5	−13.3 ± 0.7*	−18.0 ± 1.5*
	10	138.8 ± 1.7	0.8 ± 2.3	−0.3 ± 1.8	−10.5 ± 1.9	−13.6 ± 1.9*
Protein extract						
C. tomentosum	500	147.2 ± 3.9	−8.8 ± 2.0*	2.3 ± 1.7	−5.1 ± 3.9	8.2 ± 2.8
	900	149.5 ± 1.4	−7.1 ± 3.7*	2.3 ± 3.8	−8.9 ± 1.8	−9.3 ± 2.4
H. elongata	100	135.8 ± 1.7	−1.1 ± 1.3	2.7 ± 1.7	−4.4 ± 2.9	−11.6 ± 2.9
	200	135.6 ± 2.8	−3.3 ± 1.8*	−4.9 ± 1.6	−16.2 ± 2.8*	−17.7 ± 2.5*
	400	136.1 ± 3.4	−4.8 ± 3.4	−5.9 ± 2.7	−16.1 ± 2.6*	−18.2 ± 1.9*

Each value represents the mean ± S.E.M. of 6–10 rabbits.
*Significantly different from control, $P < 0.01$.

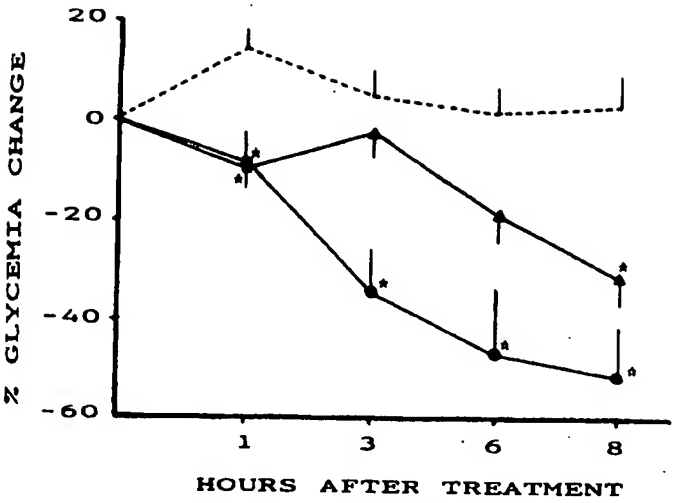

Fig. 1. Effects of *H. elongata* extracts on alloxan-diabetic rabbits. (– – –, Control; —▲—, 200 mg/kg protein extract; —●—, 5 mg/kg polysaccharide). Each point represents the mean ± S.E.M. of 5–9 rabbits. *Significantly different from control, $P < 0.01$.

In view of the evident hypoglycemiant effect of the *H. elongata* extracts on normoglycemic animals, 5 mg/kg doses of crude polysaccharide and 200 mg/kg of protein solution were assayed on rabbits with alloxan-induced diabetes whose initial fasting glycemia levels ranged from 304 to 572 mg%. Hypoglycemiant effects similar to those found in normal animals were observed, with +8 h reductions of 31.5% and 51.4% being caused by the protein and polysaccharide respectively (Fig. 1).

Pending further experimentation, the mechanism of the hypoglycemiant activity of the *H. elongata* extracts can only be conjectured (an effect on insulin secretion, for example, could be revealed by monitoring insulin levels after administration of the extracts). It seems likely, however, that the activity of the polysaccharide extract may have the same mechanism as various polysaccharides isolated from terrestrial plants (Tomoda et al., 1986), while that of the protein solution may be similar to that of the hypoglycemiant protein fraction isolated by Güven and Güler (1979) from the red alga *Pterocladia capillacea*. We are currently continuing our work on *H. elongata* with a view to answering these questions.

Acknowledgements

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Note

α-Glucosidase Inhibitory Activity of a 70% Methanol Extract from Ezoishige (*Pelvetia babingtonii* de Toni) and Its Effect on the Elevation of Blood Glucose Level in Rats

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The 70% methanol extract from ezoishige (*Pelvetia babingtonii* de Toni) inhibited the rat-intestinal α-glucosidase, sucrase and maltase activities, with IC_{50} values of 2.24 and 2.84 mg/ml. Sucrose was orally administered with or without the extract to rats at 1000 mg/kg. The postprandial elevation in the blood glucose level at 15 and 30 min after the administration of sucrose with the extract was significantly suppressed when compared with the control. These results suggest that the extract from ezoishige has potent α-glucosidase inhibitors and would be effective for suppressing postprandial hyperglycemia.

Key words: inhibitor; α-glucosidase; *Pelvetia babingtonii* de Toni

The frequency of diabetes, obesity, and hyperlipemia in the population worldwide is high and still increasing. In the course of these metabolic disorders, the concentrations of blood glucose, serum insulin, and lipoproteins each differ from the normal, especially after food intake. A reasonable way to control these carbohydrate-dependent diseases would be to limit intestinal carbohydrate digestion. Intestinal α-glucosidase (EC 3.2.1.20) plays an important role in carbohydrate digestion and absorption. Therefore, an inhibitor of intestinal α-glucosidase could be expected to retard carbohydrate digestion and absorption. Potent α-glucosidase inhibitors such as acarbose[1] and voglibose[2] have already been clinically used as medicines for diabetic and obese patients.

Great interest is currently being devoted to the physiological functions of food components relating to the prevention of diabetes and obesity. In previous *in vitro* studies, α-glucosidase inhibitors have been isolated from various food materials, *e.g.*, ougon,[3] hijiki,[4] tochu-cha,[5] welsh onion,[6] and clove.[7] However, the *in vitro* inhibitory activity is not always related to the *in vivo* activity for physiological action. To utilize food materials as physiological modulators, it is necessary to confirm the *in vivo* action of such materials following their oral administration.

In this present study, an extract of the brown alga, ezoishige (*Pelvetia babingtonii* de Toni), was examined for its *in vitro* inhibition of rat-intestinal α-glucosidase and its *in vivo* effect on the elevation of blood glucose in rats.

The *in vitro* α-glucosidase inhibition test was performed by using a crude α-glucosidase solution prepared from rat-intestinal powder. Rat-intestinal acetone powder was purchased from Sigma Chemical Co. (St. Louis, Mo, U.S.A). Sucrose and maltose were purchased from Kanto Kagaku Industry Co. (Tokyo, Japan), and ezoishige was collected from the coast of Mitsuishi, Hokkaido Prefecture in Japan. Ezoishige (100 g) was homogenized with 70% methanol (500 ml) by a Polytron device (Kinematica PT-6000, Littau, Switzerland) for 1 min at room temperature. The resulting homogenate was centrifuged at 10,000 × *g* for 10 min, and then the supernatant was passed through No. 5B filter paper. The filtrate was evaporated and dried under reduced pressure, before being dissolved in 70% methanol and used for the assay of α-glucosidase inhibitory activity. The inhibitory activity toward rat-intestinal α-glucosidase was measured by a slightly modified method of Asano *et al.*[8] One gram of rat-intestinal acetone powder was suspended in 10 ml of 0.9% saline, and the suspension sonicated (1 min × 3). After centrifugation (3,000 rpm × 30 min), the resulting supernatant was used for the assay. A crude α-glucosidase solution showed specific activities against maltase (1.94 units/mg of protein) and sucrase (0.42 units/mg of protein) which were measured by using sucrose and maltose as substrates. The assay mixture consisted of a 100 mM maleate buffer (pH 6.0, 0.7 ml), 500 mM sucrose or 500 mM maltose (0.1 ml), and the sample extract in 70% methanol (0–167.0 mg/ml, 0.1 ml). Methanol did not affect the enzyme activity under these conditions. The mixture

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was preincubated for 5 min at 37°C, and the reaction was initiated by adding a crude α-glucosidase solution (0.1 ml) to the reaction mixture. This mixture was incubated for 60 min at 37°C, the reaction being terminated by adding 1.0 ml of a 2.0 M maleate-Tris-NaOH buffer (pH 7.4). The glucose released in the reaction mixture was determined by a Glucose C-II Test Wako kit (Wako Pure Chemical Co., Tokyo, Japan) based on the mutarotase-glucose oxidase method. The reaction mixture (0.02 ml) and Glucose C-II Test Wako kit (3.0 ml) were mixed and incubated for 20 min at 37°C, before the absorbance of the mixture was measured at 505 nm.

Seven-week-old male Wistar strain rats were purchased from Nippon SLC Co. (Tokyo, Japan), rats weighing approximately 163–191 g being used. The animals were fed on standard feed (Labo MR stock, Nippon Nosan Kogyo Co., Tokyo, Japan) and tap water *ad libitum*. Each animal was housed in a cage under controlled temperature (22±3°C) and humidity (50±20%) with a cycle of 12 h for lights on and off. The animal experiments in this study were performed under the guidelines for animal experiments according to Notification No. 6 of the Japanese government.

The rats were used for the oral administration experiment after food deprivation for 12 h. The dried extract prepared as already described was dissolved in distilled water, and a sucrose solution (500 mg/kg) was orally administered to the rats with or without the extract (1,000 mg/kg) by using a zonde. Blood samples (0.5 ml) were collected before and 15, 30, 60 and 120 min after administration from the jugular vein of each rat under ether anesthesia. The plasma was separated from the collected blood, and the concentration of glucose was measured by the Glucose C-II Test Wako kit. The significance of differences in the glucose level against the control (sucrose alone) was analyzed by Student's *t*-test.

The results show that the 70% methanol extract from ezoishige had dose-dependent inhibitory activity against both sucrase and maltase with IC_{50} values of 2.24 and 2.84 mg/ml, respectively (Fig. 1). To evaluate the *in vivo* action, animal experiments were conducted with a single oral administration together with sucrose to the rats. Figure 2 shows the effect of the 70% methanol extract from ezoishige on the blood glucose level after orally administrating with sucrose to the rats. After the administration, the blood glucose level in the rats of the control group (sucrose alone) showed a maximum value after 15 min, before falling moderately after 30 min. The extract from ezoishige, however, significantly ($P<0.01$) depressed the postprandial elevation in blood glucose compared with the control group during the 15–30 min period after sucrose loading. The blood glucose level of the extract administered rats was identical to the level in control group during the

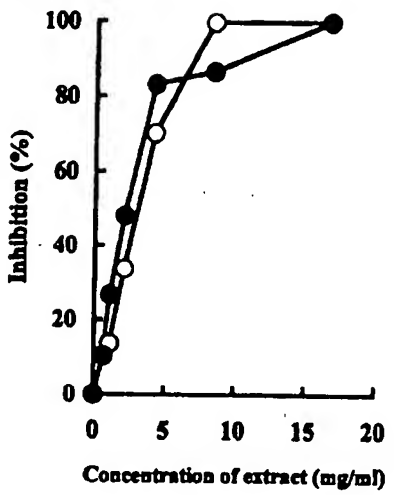

Fig. 1. Inhibitory Activity of the 70% Methanol Extract from Ezoishige against Sucrase and Maltase in Rat Intestine.
○ sucrase activity; ● maltase activity

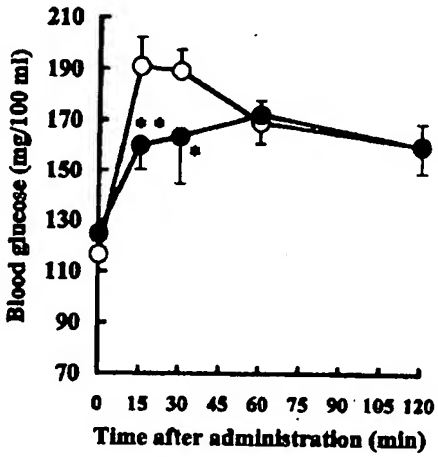

Fig. 2. Effect of the 70% Methanol Extract from Ezoishige on the Blood Glucose Level after an Oral Administration of Sucrose to Rats.

Rats were administered with sucrose (500 mg/kg) alone or with the extract (1,000 mg/kg). Each point represents the mean ±S.E. ($n=10$). Significant difference in glucose level against that of the corresponding control: $^{*}P<0.01$, $^{**}P<0.001$.
○ sucrose; ● sucrose + extract

period from 60 to 120 min. These results show that the extract had a suppressive effect on the postprandial elevation in blood glucose after its oral administration to rats.

Although marine algae are increasingly being investigated for their novel and potentially bioactive components,[9] there have been no reports on the inhibitory activity of animal α-glucosidase and its *in vivo* action after an oral administration to rats. Most of the *in vitro* studies on food materials have used α-glucosidase from bakers yeast. Compared with the

previously reported inhibitory substances in foods[10] that have been evaluated *in vitro* by using yeast α-glucosidase, the extract obtained in this study showed the most potent inhibitory activity. Furthermore, the extract from ezoishige significantly suppressed the postprandial elevation in blood glucose after an oral sucrose loading in rats. The present results demonstrate that the extract from ezoishige contained potent α-glucosidase inhibitors and was effective for suppressing postprandial hyperglycemia. Ezoishige, which is an underutilized brown alga, is therefore considered to be a promising functional food material for controlling the blood glucose level to prevent and/or reduce the risk of diabetes and obesity. Purification and isolation of the α-glucosidase inhibitors in the extract from ezoishige are now in progress.

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Anti-obesity Action of *Salix matsudana* Leaves (Part 1). Anti-obesity Action by Polyphenols of *Salix matsudana* in High Fat-diet Treated Rodent Animals

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In preliminary experiments, polyphenol fractions prepared from the leaves of *Salix matsudana* reduced the elevation of the rat plasma triacylglycerol level at 3 and 4 h after oral administration of a lipid emulsion containing corn oil, at a dose of 570 mg/kg. The present study examined the anti-obesity action of polyphenol fractions of *S. matsudana* leaves by testing whether the polyphenol fractions prevented the obesity induced by feeding a high-fat diet to female mice for 9 weeks. Body weights at 2–9 weeks and the final parametrial adipose tissue weights were significantly lower in mice fed the high-fat diet with 5% polyphenols of *S. matsudana* leaves than in those fed the high-fat diet alone. The polyphenols of *S. matsudana* leaves also significantly reduced the hepatic total cholesterol content, which was elevated in mice fed the high-fat diet alone. In addition, the polyphenol fractions of *S. matsudana* leaves inhibited palmitic acid uptake into brush border membrane vesicles prepared from rat jejunum and α-amylase activity, and their fractions enhanced norepinephrine-induced lipolysis in fat cells. In conclusion, it is suggested that the inhibitory effects of the flavonoid glycoside fraction of *S. matsudana* leaves on high-fat diet-induced obesity might be due to the inhibition of carbohydrate and lipid absorption from small intestine through the inhibition of α-amylase and palmitic acid uptake into small intestinal brush border membrane or by accelerating fat mobilization through enhancing norepinephrine-induced lipolysis in fat cells. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: Salix matsudana; α-amylase activity; brush border membrane vesicles; high-fat diet; parametrial adipose tissue weight.

INTRODUCTION

Salix matsudana Koidz (Berberidaceae) 'Hanliu' in Chinese, is mostly distributed in northern and western China, and its leaves have been used for more than 3000 years as a traditional Chinese folk drug for treating jaundice, hepatitis, rheumatism, arthritis and eczema (Jiangsu New Medical College, 1977). Though it has recently been reported that the leaves of *S. matsudana* have anti-obesity actions, the basis for this hearsay is unclear. In preliminary experiments, it was found that polyphenol fractions of *S. matsudana* leaves appeared to reduce the elevation of plasma triacylglycerol after oral administration of a lipid emulsion consisting of corn oil, cholic acid, cholesteryloleate and saline solution. Therefore, the fat balance was measured by determination of fat excretion in the faeces of mice fed a high-fat diet or a high-fat diet plus the polyphenol fraction of *S. matsudana* leaves for 3 days, and the effects of polyphenol fraction on obesity induced by high-fat diet over the long term were examined. In addition, the anti-obesity mechanisms of polyphenol fraction of the leaves of *S. matsudana* were investigated using a lipolytic assay in rat adipocytes and an assay for inhibition of α-amylase activity *in vitro*, and inhibition of fatty acid uptake into brush border membrane vesicles prepared from rat jejunum.

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Contract/grant sponsor: Tachibana Co. Ltd (Tokyo, Japan).

MATERIALS AND METHODS

Materials. The [1-^{14}C] palmitic acid was obtained from Du Pont NEN (England). Norepinephrine was purchased from Daiichi Pharmacy Co. (Tokyo, Japan). Collagenase (type IV) was purchased from Worthington Biochemical Co. (Freehold, NJ), and bovine serum albumin (BSA) was purchased from Wako Pure Chemical Co. (Osaka, Japan) and was extracted by the method of Chen (1967) to remove free fatty acids. The triglyceride E-test and total cholesterol E-test kits were purchased from Wako Pure Chemical Co. (Osaka, Japan). Amylase was obtained from Sigma (St Louis, MO). Sephadex LH-20 was purchased from Pharmacia Biotech Co. (Sweden). Other chemicals were of reagent grade.

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Table 1. Composition of experimental diets[a]

	High fat diet	High fat diet plus 2% polyphenol fractions (g/100 g food)	High fat diet plus 5% polyphenol fractions
Beef tallow	40	40	40
Corn starch	10	10	10
Sugar	9	9	9
Mineral mixture	4	4	4
Vitamin mixture	1	1	1
Casein	36	34	31
Polyphenol fractions of *Salix matsudana*[b]	0	2	5
kcal/100 g diet	580	580	580

[a] Laboratory pellet chow: water 8 g, carbohydrate 51.3 g, protein 24.6 g, lipid 5.6 g, fibre 3.1 g, mineral mixture 6.4 g and vitamin mixture 1 g per 100 g food; 355 kcal/100 g diet.
[b] Polyphenol fractions were calculated as 4 kcal.

Plant materials. The leaves of *Salix matsudana* were obtained from Jilin Sheng in China and voucher samples are stored at the Second Department of Medical Biochemistry, School of Medicine, Ehime University.

Animals. Male Wistar King strain rats (5 weeks old) and female ICR strain mice (3 weeks old) were purchased from Charles River Japan (Yokohama, Japan) and CLEA Japan Inc. (Osaka, Japan), respectively, and housed for 1 week in a 12 h/12 h light/dark cycle in a temperature- and humidity-controlled room. The animals were given free access to food and water. After adaptation to the lighting conditions for 1 week, the healthy animals were used in the present experiments. The Animal Studies Committee of Ehime University approved the experimental protocol.

Estimation of plasma triacylglycerol after oral administration of lipid emulsion in rats. Male Wistar King strain rats (7 weeks old, body weight 250 g) that had fasted overnight were orally administered 3 mL of lipid emulsion consisting of corn oil (6 mL), cholic acid (80 mg), cholesteryloleate (2 g) and saline solution (6 mL) with or without the polyphenol fraction of *S. matsudana* leaves (575 mg/kg body weight). Blood was taken from the tail vein 0, 1, 2, 3 and 4 h after the oral administration of the lipid emulsion with or without the polyphenol fractions of *S. matsudana* leaves and centrifuged at 5500 × *g* for 5 min using a model KH-120 M centrifuge (Kubota, Tokyo, Japan) to obtain the plasma. The triacylglycerol was determined using a triglyceride E-test Wako kit.

Estimation of fat excretion in faeces of mice fed a high-fat diet for 3 days. The mice in the experimental groups received the high-fat diet containing 2% or 5% polyphenol fraction for 3 days. The food intake of each mouse was estimated every day, and samples of faeces were obtained from each animal at regular intervals. The triacylglycerol and total cholesterol contents in faeces were measured by extraction of 150 mg sample with $CHCl_3$–MeOH (2:1, v/v, 4 mL), the extract being concentrated under a nitrogen stream. The fat content of the residue was determined using triglyceride and total cholesterol E-Test Wako kits, respectively.

Estimation of body weight, parametrial adipose tissue weight, adipose cell size, liver weight, and hepatic triacylglycerol and total cholesterol contents. Female ICR mice (3 weeks old) were maintained in a 12 h/12 h light/dark cycle in a temperature- and humidity-controlled room. The animals were fed laboratory pellet chow (CLEA Japan Inc., Osaka, Japan) and given water *ad libitum*. The healthy mice were divided into four groups (n = 14) with each group matched for body weight after 1 week of feeding. The control mice continued to be fed the laboratory pellet chow *ad libitum*. The basic composition of the experimental diet was as follows (g/100 g food): beef tallow 40, corn starch 10, sugar 9, vitamin mixture 1 and mineral mixture 4. The composition of the diet for each experimental group is shown in Table 1. Previously it was reported that reduction of casein in the high-fat diet from 36% to 31% did not affect either body weight or parametrial adipose tissue weight (Han *et al.*, 1999). Based on these facts, the flavonoid glycoside fractions of *S. matsudana* leaves was added to the high-fat diet instead of casein. To avoid auto-oxidation of their fat contents, the feeds were stored at −30 °C and freshly prepared each day. Each mouse was weighed once a week and the weight recorded. The total amount of food intake by each mouse was recorded at least three times a week. After 9 weeks of consuming the indicated experimental diet, the blood of each mouse was taken by venous puncture under anaesthetization with diethyl ether, and then the mouse was killed with an overdose of diethyl ether administered for about 2 min. The livers and parametrial adipose tissues were quickly removed and weighed, and liver tissues were stored at −80 °C until analysis. The adipose tissue shreds, each 100 mg in weight, were immediately plunged into a plastic tube containing 1.5 mL of 2% osmium tetroxide in 0.05 M collidine–HCl buffer at pH 7.4, and the tissues were fixed at 37 °C for 72 h. After fixation, the contents of the plastic container were thoroughly washed through a nylon screen (250 μm) with distilled water. The filtrate contained most of the fixed free cells, but fibrous tissue and some intact shreds of fixed adipose tissue remained on the filter. The tissue shreds were gently rubbed by hand on the filter, and the washing was continued. This procedure completely separated the tissue into free cells,

and resulted in total recovery of cells in the filtrate. These cells were then collected and washed with distilled water by using a finer screen (25 μm). The diameter of adipose cells was determined by examining osmium tetroxide-fixed cells by scanning electron micrography (Hitachi H-500). The liver triacylglycerol contents were measured as follows: a portion (0.5 g) of the liver tissue was homogenized in Krebs Ringer phosphate buffer (pH 7.4, 4.5 mL), the homogenate (0.2 mL) was extracted with $CHCl_3$–MeOH (2:1, v/v, 4 mL), and the extract was concentrated under a nitrogen stream. The residue was analysed using Triglyceride E-test and Total Cholesterol E-test Wako kits.

Preparation of polyphenol fractions from *S. matsudana* leaves. The dry leaves (5 kg) of *S. matsudana* were extracted with petroleum ether (12 L × 2 times) for 3 h under reflux. The combined petroleum ether extracts were concentrated *in vacuo* to give a dark reddish brown extract (78 g). The residue was extracted with 95% EtOH (10 L × 3 times) for 3 h under reflux. The combined 95% EtOH extracts were concentrated *in vacuo* to give a brown extract (655 g). A portion (500 g) of the 95% EtOH extract was suspended in distilled water (2.5 L), extracted with benzene (1 L × 3 times), CH_2Cl_2 (1 L × 3 times), EtOAc (1 L × 3 times), *n*-BuOH (1 L × 3 times) and divided into benzene-, CH_2Cl_2-, EtOAc-, and *n*-BuOH-soluble and -insoluble fractions, which were concentrated *in vacuo* to give 47 g, 142 g, 33 g, 141 g, 235 g residues, respectively. The *n*-BuOH-soluble fraction (10 g) was suspended in MeOH, and chromatographed over Sephadex LH-20 (column: 1.5 × 90 cm) and developed with MeOH. The MeOH extract gave a red colour by adding Mg powder and 0.5 N HCl and a dark blue colour by adding $FeCl_3$. Therefore, these fractions were named as polyphenol fractions.

Preparation of fat cells. Young male Wistar rats were killed by cervical dislocation, and their epididymal adipose tissue was quickly removed. Fat cells were isolated from the adipose tissue by the method of Rodbell (1964).

Measurement of norepinephrine-induced lipolysis in fat cells. An aliquot of the fat cell fraction (50 μL packed volume) was incubated for 1 h at 37 °C in 200 μL of Hanks balanced solution (pH 7.4) supplemented with 2.5% BSA, norepinephrine (25 μL, final concentration: 0.05 μg/mL) and the indicated amounts of test compounds (25 μL). The release of free fatty acid (FFA) was measured as described previously (Okuda *et al.*, 1986). Briefly, the incubation mixture (250 μL) was mixed with 3 mL of chloroform/*n*-heptane (1:1, v/v) containing 2% methanol, and extracted by shaking the tube horizontally for 10 min in a shaker. The mixture was centrifuged at 2000 × *g* at 25 °C for 5 min, and the upper aqueous phase was removed by suction, and copper reagent (1 mL) was added to the lower organic phase. Then the tube was shaken for 10 min, the mixture was centrifuged at 2000 × *g* at 25 °C for 10 min, and 0.5 mL of the upper organic phase (which contained the copper salts of the extracted fatty acid) was treated with 0.5 mL of 0.1% (w/v) bathcurproine in chloroform containing 0.05% (w/v) 3-(2)-tertbutyl-4-hydroxyanisol. The absorbance of the solution was then measured at 480 nm. Lipolysis was expressed as μmole of FFA released per mL of packed fat cells per h.

Measurement of α-amylase activity *in vitro*. Soluble starch (0.4 mg/mL) in 0.25 M phosphate buffer (pH 7.0) was used as the substrate. The assay system contained the following components in a total volume of 2.02 mL: 1.0 mL substrate solution, 1.0 mL test compound solution and 0.02 mL amylase solution (final concentration 0.03 μg/mL). Incubation was carried out at pH 7.0 and 37 °C for 7.5 min. The mixture was treated with 1.0 mL of 0.01 N I_2 solution and 5.0 mL of distilled water. Its absorbance was then measured at 660 nm.

Lipid absorption by brush border membrane vesicles. Brush border membrane vesicles were prepared from the jejunum portion of the rat small intestine according to the method of Kessler *et al.* (1978). Donor vesicles composed of egg PC/palmitic acid (93:7 molar ratio) and a trace of [1-^{14}C] palmitic acid were made by sonicating 1.0 mL of the mixed lipid dispersion in 10 mM HEPES-Tris buffer, pH 7.5, containing 100 mM mannitol (buffer A) for 5 min at 4 °C. The assay mixture for investigating lipid absorption consisted of 0.24 mL of buffer A containing 29 nmol of palmitic acid (160 000 dpm) and the brush border membrane vesicles (48 μg of protein). Incubation was carried out for 30 min at 20 °C. After incubation, 0.1 mL of the incubation medium was diluted with 1 mL of ice-cold buffer A, and this solution was immediately filtered through 0.45 μm cellulose nitrate filters and washed four times with 1 mL of ice-cold buffer A. The filters were then dissolved as recommended by the supplier of ACS II, and their radioactivity was measured.

Statistical analysis. The results are expressed as mean ± standard error (SEM). Data were analysed by one-way analysis of variance (ANOVA), and then differences in mean values among groups were analysed using Fisher's protected LSD multiple comparison test and were considered significantly different at $p < 0.05$.

RESULTS AND DISCUSSION

Plasma triacylglycerol level after oral administration of lipid emulsion in rats

Figure 1 shows the time course of the plasma triacylglycerol level after oral administration of lipid emulsion. At 3 h and 4 h after oral administration of lipid emulsion, the polyphenol fractions of *S. matsudana* leaves significantly reduced the plasma triacylglycerol level. Elevation of plasma total cholesterol was caused by oral administration of lipid emulsion. The polyphenol fractions did not affect the plasma total cholesterol level after oral administration of lipid emulsion (data not shown).

Faecal triacylglycerol and total cholesterol in mice fed a high-fat diet for 3 days

The consumption of a high-fat diet plus the polyphenol fractions of *S. matsudana* leaves enhanced triacylglycerol and total cholesterol excretion in the faeces and inhibited absorption of the ingested dietary fat (Table 2).

Table 2. Effects of the polyphenol fractions of *S. matsudana* leaves on triacylglycerol and total cholesterol excretion into faeces of mice fed a high-fat diet for 3 days

	Triacylglycerol (μmol/total faeces/day)	Total cholesterol (μmol/total faeces/day)
Normal mice	5.04 ± 0.40*	13.73 ± 0.91*
High-fat diet group	3.42 ± 0.23	4.19 ± 0.54
High-fat plus 2% polyphenol fraction group	3.17 ± 1.02	5.82 ± 0.31
High-fat plus 5% polyphenol fraction group	6.68 ± 0.49*	6.88 ± 0.47*

Results are expressed as the mean ± SEM, $n = 4$. * $p < 0.05$, significantly different from a high-fat diet group.

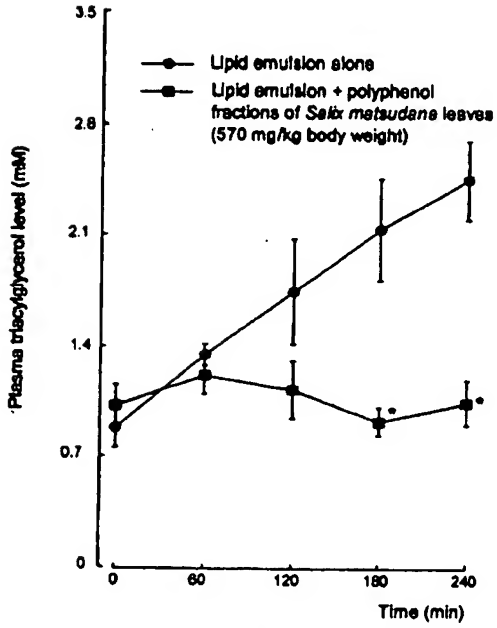

Figure 1. Effects of polyphenol fractions of *S. matsudana* leaves on rat plasma triacylglycerol level after oral administration of a lipid emulsion. Each point represents the mean ± SEM, $n = 4$. * $p < 0.05$, significantly different from a high-fat diet group.

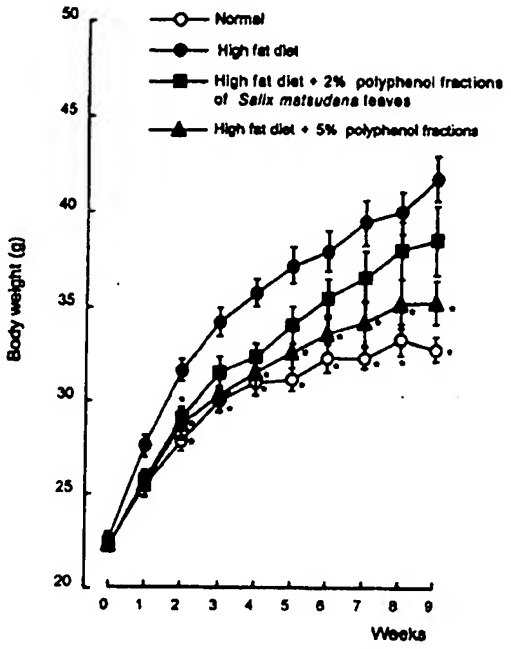

Figure 2. Effects of polyphenol fractions of *S. matsudana* leaves on body weight in mice fed a high-fat diet for 9 weeks. Results are expressed as the mean ± SEM, $n = 14$. * $p < 0.05$, significantly different from a high-fat diet group.

Body, parametrial adipose tissue and liver weights, liver triacylglycerol and total cholesterol, food consumption and adipose cell size in mice fed a high-fat diet for 9 weeks

Figure 2 shows the changes in body weight of the groups during the experiment. Consumption of a high-fat diet containing 40% beef tallow for 9 weeks produced significant increases in body weight and parametrial adipose tissue weight compared with consumption of laboratory pellet chow (control group) (Figs 2 and 3). Furthermore, the high-fat diet also induced fatty liver with an accumulation of triacylglycerol and total cholesterol compared with the control group (Table 3). Consumption of a high-fat diet containing 5% polyphenol fractions of *S. matsudana* significantly reduced the increases in body and final parametrial adipose tissue weights compared with those in the high-fat diet group. Consumption of a high-fat diet containing 2% polyphenol fractions also tended to reduce the increases in body and final parametrial adipose tissue weights, but the differences were not significant. Consumption of a high-fat diet plus polyphenol fractions significantly reduced the hepatic total cholesterol content, and also tended to reduce the hepatic triacylglycerol content, although the difference was not significant compared with the high-fat diet group (Table 3). The rate of reduction in body weigh corresponded to that of the reduction in parametrial adipose tissue weight. The mean food consumption per week per mouse during the whole experimental period was significantly different between the laboratory chow diet and high-fat diet groups, being 424.2 ± 4.6 kJ/week/mouse in the laboratory chow diet group and 578.3 ± 15.4 kJ/week/mouse in the high-fat diet group. There was no significant difference in food consumption between the high-fat diet group (578.3 ± 15.4 kJ/week/mouse) and the high-fat diet plus 2% polyphenol fractions group (570.1 ± 20.6 kJ/week/mouse) or high-fat diet plus 5%

Table 3. Effects of the polyphenol fractions of *S. matsudana* leaves on liver weight, hepatic triacylglycerol and total cholesterol content in mice fed a high-fat diet for 9 weeks

	Liver weight (g/100 g body weight)	Triacylglycerol (μmol/g liver)	Total cholesterol (μmol/g liver)
Normal mice	4.8 ± 0.15*	18.9 ± 1.1*	7.5 ± 0.26*
High-fat diet group	6.0 ± 0.25	164.8 ± 8.9	14.5 ± 0.36
High-fat plus 2% polyphenol fraction group	5.9 ± 0.26	143.5 ± 14.0	10.6 ± 0.36*
High-fat plus 5% polyphenol fraction group	5.7 ± 0.21	138.6 ± 13.6*	10.1 ± 0.39*

Results are expressed as the mean ± SEM, $n = 14$. * $p < 0.05$, significantly different from a high-fat diet group.

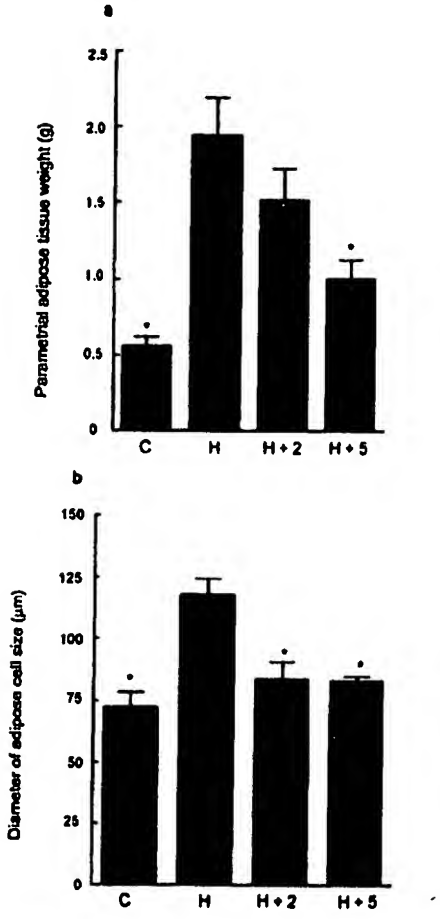

Figure 3. Effects of polyphenol fractions of *S. matsudana* leaves on parametrial adipose tissue weight (a) and diameter of adipose cells (b) in mice fed a high-fat diet for 9 weeks. C, control group; H, high-fat diet; H + 2, high-fat diet plus 2% polyphenol fractions of *S. matsudana* leaves; H + 5, high-fat diet plus 5% polyphenol fractions. Results are expressed as the mean ± SEM, $n = 14$. * $p < 0.05$, significantly different from a high-fat diet group.

polyphenol fractions group (571.0 ± 19.4 kJ/week/mouse). The diameter of adipose cells was significantly greater in the high-fat diet group than that in the control group, and the diameter in the high-fat diet plus polyphenol fraction-treated group was increased less than that in the group fed the high-fat diet only (Fig. 3).

Table 4. Effects of 95% EtOH and polyphenol fraction of *S. matsudana* leaves on norepinehrine-induced lipolysis in isolated fat cells

Addition (/mL reaction mixture)	% of control
None	0 ± 0*
Norepinephrine (Norepi) (0.05 μg)	100.0 ± 1.5
Norepi + 95% EtOH extract (1 mg)	131.9 ± 2.4*
Norepi + Non-polyphenol fraction (1 mg)	95.3 ± 5.0
Norepi + Polyphenol fraction (1 mg)	126.1 ± 2.5*

Results are expressed as the mean ± SEM, $n = 4$. * $p < 0.05$, significantly different from norepinephrine alone.

Norepinephrine-induced lipolysis

The 95% EtOH extract of *S. matsudana* leaves enhanced norepinephrine-induced lipolysis at a concentration of 1 mg/mL (Table 4), while it did not cause lipolysis in the absence of norepinephrine (data not shown). The *n*-BuOH-soluble fraction separated from the 95% EtOH extract and polyphenol fractions prepared from *n*-BuOH-soluble fraction also enhanced norepinephrine-induced lipolysis at a concentration of 1 mg/mL, but non-polyphenol fractions had no effect on norepinephrine-induced lipolysis.

α-Amylase activity by flavonoid glycoside fractions

It has been reported that a α-amylase inhibitor from wheat flour prevented obesity through inhibition of digestion and absorption of carbohydrates (Yokota *et al.*, 1994; Lankisch *et al.*, 1998). It was found that the polyphenol fractions inhibited amylase activity at concentrations of 250–5000 μg/mL (Table 5).

Table 5. Effects of polyphenol fractions of *S. matsudana* leaves on α-amylase activity

Addition (/mL reaction mixture)	α-Amylase activity (% of control)
None	100.0 ± 2.5
Polyphenol fractions (250 μg)	98.2 ± 0.8
(2500 μg)	61.3 ± 1.1*
(5000 μg)	20.0 ± 0.5*

Results are expressed as the mean ± SEM, $n = 4$–8. * $p < 0.05$, significantly different from no addition (none).

Table 6. Effects of polyphenol fractions of *S. matsudana* leaves on palmitic acid uptake into brush border membrane vesicles of rat jejunum

Addition (/mL reaction mixture)	Palmitic acid uptake to small intestinal brush border membrane (% of control)
None	100.0
Polyphenol fractions (250 μg)	81.4
(500 μg)	0.0
(1000 μg)	0.0

Results are expressed as the mean, $n = 2$.

Palmitic acid uptake into brush border membrane vesicles of rat small intestine *in vitro*

The polyphenol fractions completely inhibited the incorporation of palmitic acid into brush border membrane vesicles at concentrations of 500 and 1000 μg/mL (Table 6).

There are a number of studies describing high-fat diet-induced obesity (Flatt, 1987; Awad *et al.*, 1990; Shimomura *et al.*, 1990; Hill *et al.*, 1993). Though it has recently been reported that the leaves of *S. matsudana* have anti-obesity actions, the basis for this hearsay is unclear. Therefore, experiments were designed to clarify whether high-fat diet-induced obesity in female mice may be prevented by *S. matsudana* leaves, possibly due to inhibition of the intestinal absorption of dietary fat and carbohydrates. In the present study, it was found that polyphenol fractions of *S. matsudana* leaves prevented the increases in body and parametrial adipose tissue weights in mice fed a high-fat diet containing 40% beef tallow for 9 weeks. The high-fat diet caused the accumulation of liver triacylglycerol and total cholesterol compared with the control group. The hepatic total cholesterol content was reduced by the administration of the polyphenol fractions compared with the high-fat diet-treated group, and the hepatic triacylglycerol content was also reduced but not significantly. On the other hand, the liver weight was nearly the same between the high-fat diet and high-fat diet plus polyphenol fraction-treated groups. The mean food consumption per week per mouse during the whole experimental period was significantly different between the laboratory chow and high-fat diet groups, but not significantly different between the high-fat diet and high-fat diet plus polyphenol fraction-treated groups. These results suggest that the polyphenol fractions of *S. matsudana* leaves might exert their anti-obesity action through inhibition of intestinal absorption of dietary fat and carbohydrates, acceleration of lipolysis in adipose tissue, and by other mechanisms. It is well known that dietary fat is not absorbed from the intestine unless it has been subjected to the action of pancreatic lipase (Verger, 1984). Polyphenol fractions of *S. matsudana* leaves did not affect pancreatic lipase activity *in vitro* (data not shown), whereas they inhibited the absorption of palmitic acid by small intestinal brush border membrane vesicles. These results suggest that the polyphenol fractions of *S. matsudana* leaves may reduce the intestinal absorption of dietary fat by inhibiting the absorption of palmitic acid, one of the products of hydrolysis of dietary fat. In fact, the polyphenol fractions were confirmed to significantly reduce the plasma triacylglycerol level that was elevated due to oral administration of a lipid emulsion containing corn oil. These results suggest that the reduction of plasma triacylglycerol levels by the polyphenol fractions of *S. matsudana* leaves may be mediated through inhibition of the intestinal absorption of palmitic acid produced by the hydrolysis of corn oil. The accumulation of fat in the liver and in parametrial adipose tissue induced by a high-fat diet was reduced by the consumption of the polyphenol fractions, perhaps through inhibition of the intestinal absorption of carbohydrate by the inhibition of α-amylase, or through stimulation of excretion of ingested dietary fat in the faeces, or the stimulation by polyphenol fractions of norepinephrine-induced lipolysis in adipocytes. These results conclude that the inhibitory effects of polyphenol fraction of *S. matsudana* leaves on high-fat diet-induced obesity might be due to the inhibition of carbohydrate and lipid absorption from the small intestine through the inhibition of α-amylase and palmitic acid uptake into small intestinal brush border membrane or accelerating fat mobilization through enhancing norepinephrine-induced lipolysis in fat cells.

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Anti-obesity Action of *Salix matsudana* Leaves (Part 2). Isolation of Anti-obesity Effectors from Polyphenol Fractions of *Salix matsudana*

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Previously, it was reported that polyphenol fractions prepared from the leaves of *Salix matsudana* reduced the elevation of the rat plasma triacylglycerol level at 3 and 4 h after oral administration of a lipid emulsion containing corn oil, at a dose of 570 mg/kg. Moreover, body weights at 2–9 weeks and the final parametrial adipose tissue weights were significantly lower in mice fed the high-fat diet with 5% polyphenol fractions of *S. matsudana* leaves than in those fed the high-fat diet alone. The polyphenol fractions of *S. matsudana* leaves also significantly reduced the hepatic total cholesterol content, which was elevated in mice fed the high-fat diet alone. In addition, the polyphenol fractions of *S. matsudana* leaves inhibited palmitic acid uptake into brush border membrane vesicles prepared from rat jejunum and α-amylase activity, and their fractions enhanced norepinephrine-induced lipolysis in fat cells. To clarify the active substances inhibiting the palmitic acid uptake into small intestinal brush border membrane, the α-amylase activity or enhancing the norepinephrine-induced lipolyis in fat cells, the isolation of the active substances from polyphenol fraction was attempted using the above three assay systems. Compounds 1, 2 and 3 were isolated from the polyphenol fractions and identified as apigenin-7-*O*-D-glucoside, luteolin-7-*O*-D-glucoside and chrysoeriol-7-*O*-D-glucoside, respectively. Among three flavonoids, apigenin-7-*O*-D-glucoside inhibited α-amylase activity, and luteolin-7-*O*-D-glucoside and chrysoeriol-7-*O*-D-glucoside inhibited palmitic acid uptake into small intestinal brush border membrane. Furthermore, three flavonoid glucosides enhanced norepinephrine-induced lipolysis in fat cells. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: Salix matsudana; α-amylase activity; brush border membrane vesicles; flavonoids.

INTRODUCTION

In the previous report, it was found that polyphenol fractions of *S. matsudana* leaves prevented the increase of body weight with the increase of parametrial adipose tissue induced by feeding high-fat diet during the long term (Han *et al.*, 2003). Furthermore, the polyphenol fractions of *S. matsudana* leaves inhibited palmitic acid uptake into brush border membrane vesicles prepared from rat jejunum and α-amylase activity, and their fractions enhanced norepinephrine-induced lipolysis in fat cells (Han *et al.*, 2003). Therefore, the isolation of the anti-obesity effectors from polyphenol fractions of the leaves of *S. matsudana* was attempted using a lipolytic assay in rat adipocytes and an assay for inhibition of α-amylase activity *in vitro*, and inhibition of fatty acid uptake into brush border membrane vesicles prepared from rat jejunum.

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Contract/grant sponsor: Tachibana Co. Ltd. (Tokyo, Japan).

MATERIALS AND METHODS

Materials. The [1-^{14}C] palmitic acid was obtained from Du Pont NEN (England). Norepinephrine was purchased from Daiichi Pharmacy Co. (Tokyo, Japan). Collagenase (type IV) was purchased from Worthington Biochemical Co. (Freehold, NJ), and bovine serum albumin (BSA) was purchased from Wako Pure Chemical Co. (Osaka, Japan) and was extracted by the method of Chen (1967) to remove free fatty acids. Amylase was obtained from Sigma (St Louis, MO). Sephadex LH-20 was purchased from Pharmacia Biotech Co. (Sweden). Other chemicals were of reagent grade.

Plant material. The leaves of *Salix matsudana* were obtained from Jilin Sheng in China and voucher samples are stored at the Second Department of Medical Biochemistry, School of Medicine, Ehime University.

General experimental procedures. Proton and carbon nuclear magnetic resonance (^{1}H and ^{13}C-NMR) spectra were measured at 300 MHz and 75 Hz, respectively, on a Brucker AC-300 (Germany).

Animals. Male Wistar King strain rats (5 weeks old) were purchased from Charles River Japan (Yokohama,

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Japan), and housed for 1 week in a 12 h/12 h light/dark cycle in a temperature- and humidity-controlled room. The animals were given free access to food and water. After adaptation to the lighting conditions for 1 week, the healthy animals were used in the present experiments. The Animal Studies Committee of Ehime University approved the experimental protocol.

Preparation of fat cells and measurement of norepinephrine-induced lipolysis in fat cells. Young male Wistar rats were killed by cervical dislocation, and their epididymal adipose tissue was quickly removed. Fat cells were isolated from the adipose tissue by the method of Rodbell (1964). The measurement of norepinphrine-induced lipolysis in fat cells was performed by the same methods described in a previous report (part 1) (Han *et al.*, 2003).

Measurement of α-amylase activity and lipid absorption by brush border membrane vesicles. The measurement of α-amylase activity and lipid absorption by brush border membrane vesicles was performed by the same methods described in a previous report (part 1) (Han *et al.*, 2003).

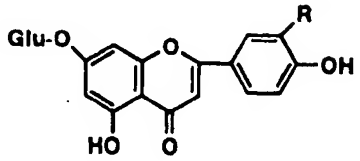
Isolation of lipolytic substances from polyphenol fractions augmenting norepinephrine-induced lipolytic activity. In the previous report, polyphenol fractions of *S. matsudana* enhanced norepinephrine-induced lipolysis in fat cells (Han *et al.*, 2003). Therefore, the polyphenol fractions (100 mg) were isolated by preparative thin layer chromatography with EtOH/*n*-BuOH/formic acid/H_2O (5:3:1:1) as eluant, to afford three active substances (compounds 1, 2 and 3) that enhanced norepinephrine-induced lipolysis. Compounds 1, 2 and 3 were identified as apigenin-7-*O*-D-glucoside, luteolin-7-*O*-D-glucoside and chrysoeriol-7-*O*-D-glucoside, respectively, by direct comparison of the ^{1}H- and ^{13}C-NMR spectral data of an authentic sample (Fig. 1). The yield of compounds 1, 2 and 3 was 22.5, 13.1 and 27.8 mg, respectively.

Statistical analysis. The results are expressed as mean ± standard error (SEM). Data were analysed by one-way analysis of variance (ANOVA), and then differences in mean values among groups were analysed using Fisher's protected LSD multiple comparison test and were considered significantly different at $p < 0.05$.

RESULTS AND DISCUSSION

In a previous report, it was found that the 95% EtOH extract of *S. matsudana* leaves, the *n*-BuOH-soluble fraction separated from the 95% EtOH extract and polyphenol fractions prepared from *n*-BuOH-soluble fraction enhanced norepinephrine-induced lipolysis at a concentration of 1 mg/mL, but non-polyphenol glycoside fractions had no effect on norepinephrine-induced lipolysis (Han *et al.*, 2003). Therefore, the isolation of the active substances from the polyphenol fractions was attempted, yielding compounds 1, 2 and 3 and identified as apigenin-7-*O*-D-glucoside, luteolin-*O*-D-glucoside and chrysoeriol-7-*O*-D-glucoside, respectively, by the comparison of their spectral data with authentic samples. Three compounds enhanced norepinephrine-induced lipolysis in fat cells (Fig. 2). These three compounds did not stimulate lipolysis in the absence of norepinephrine (data not shown). It has been reported that an α-amylase inhibitor from wheat flour prevented obesity through inhibition of digestion and absorption of carbohydrates (Yokota *et al.*, 1994; Lankisch *et al.*, 1998). Previously, it was reported that the polyphenol fractions inhibited amylase activity at concentrations of 250–5000 μg/mL. Among three isolated flavonoid glucosides, apigenin-7-*O*-D-glucoside inhibited the α-amylase activity at concentrations of 50–200 μg/mL, but luteolin-7-*O*-D-glucoside and chrysoeriol-7-*O*-D-glucoside did not (Table 1). Three isolated flavonoid glucosides inhibited palmitic acid incorporation into small intestinal brush border membrane vesicles at 25–100 μg/mL (Table 2).

Although it has recently been reported that the leaves of *S. matsudana* have anti-obesity actions, the basis for this hearsay is unclear. Previously, experiments were designed to clarify whether high-fat diet-induced obesity in female mice was prevented by *S. matsudana* leaves, possibly due to the inhibition of intestinal absorption of dietary fat and carbohydrates (Han *et al.*, 2003). It was found that polyphenol fractions of *S. matsudana* leaves prevented the increases in body and parametrial adipose tissue weights in mice fed a high-fat diet containing 40% beef tallow for 9 weeks. It seems likely that the inhibitory effects of polyphenol fraction of *S. matudana* leaves on high-fat diet-induced obesity may be due to the inhibition of carbohydrate and lipid



Compound 1= apigenin-7-*O*-D-glucoside: R=H

Compound 2= luteolin-7-*O*-D-glucoside: R=OH

Compound 3= chrysoeriol-7-*O*-D-glucoside: R=OCH_3

Figure 1. Chemical structures of three flavonoid glucosides.

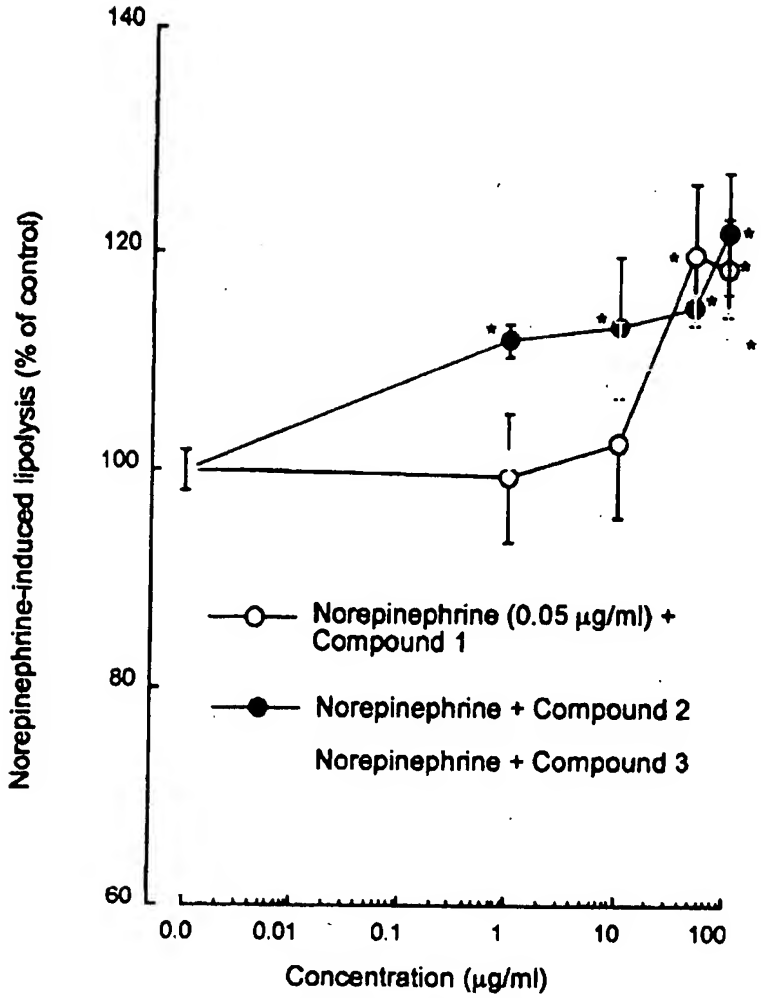


Figure 2. Effects of compounds 1, 2 and 3 isolated from polyphenol fractions of *S. matsudana* leaves on norepinephrine-induced lipolysis in fat cells. Results are expressed as the mean ± SEM, $n = 4$. The activity of norepinephrine-induced lipolysis is expressed as 100%. * $p < 0.05$, significantly different from norepinephrine alone.

Table 1. Effects of flavonoid glucosides isolated from polyphenol fractions of *S. matsudana* leaves on α-amylase activity

Addition (/mL reaction mixture)		α-Amylase activity (% of control)
None		100.0 ± 2.5
Apigenin-7-*O*-D-glucoside	(25 μg)	94.9 ± 0.1
	(50 μg)	87.3 ± 0.3*
	(100 μg)	74.6 ± 0.4*
	(200 μg)	50.6 ± 7.2*
Luteolin-7-*O*-D-glucoside	(25 μg)	99.8 ± 0.1
	(50 μg)	99.5 ± 0.1
	(100 μg)	97.8 ± 0.1
	(200 μg)	91.0 ± 0.3*
Chrysoeriol-7-*O*-D-glucoside	(25 μg)	101.2 ± 0.7
	(50 μg)	100.8 ± 0.01
	(100 μg)	99.2 ± 4.5
	(200 μg)	99.5 ± 4.1

Results are expressed as the mean ± SEM $n = 4$–8. * $p < 0.05$, significantly different from no addition (none).

Table 2. Effects of flavonoid glycosides isolated from polyphenol fractions of *S. matsudana* leaves on palmitic acid uptake into brush border membrane vesicles of rat jejunum

Addition (/mL reaction mixture)		Palmitic acid uptake to small intestinal brush border membrane (% of control)
None		100.0
Apigenin-7-*O*-D-glucoside	(25 μg)	56.1
	(50 μg)	50.0
	(100 μg)	36.9
Luteolin-7-*O*-D-glucoside	(25 μg)	66.8
	(50 μg)	48.2
	(100 μg)	22.2
Chrysoeriol-7-*O*-D-glucoside	(25 μg)	70.3
	(50 μg)	37.2
	(100 μg)	15.1

Results are expressed as the mean, $n = 2$.

absorption from the small intestine through the inhibition of α-amylase and palmitic acid uptake into small intestinal brush border membrane or accelerating fat mobilization through enhancing norepinephrine-induced lipolysis in fat cells (Han *et al.*, 2003). In the present study, apigenin-7-*O*-D-glucoside, luteolin-7-*O*-D-glucoside and chrysoeriol-7-*O*-D-glucoside were isolated from the leaves of *S. matsudana* as potential activators of norepinephrine-induced lipolysis in fat cells. Kuppusamy and Das (1992) reported the effects of flavonoids on cyclic AMP phosphodiesterase and lipid mobilization in rat adipocytes. The mechanisms by which apigenin-7-*O*-D-glucoside, luteolin-7-*O*-D-glucoside and chrysoeriol-7-*O*-D-glucoside stimulate norepinephrine-induced lipolysis are now under investigation. In addition, it was found that apigenin-7-*O*-D-glucoside isolated from *S. matsudana* leaves inhibited α-amylase activity, and that three flavonoid glucosides inhibited palmitic acid uptake into rat small intestinal brush border membrane vesicles. Therefore, it seems likely that the active substances in the anti-obesity action of the polyphenol fraction of *S. matsudana* leaves may be partly due to the flavonoid glycosides, such as apigenin-7-*O*-D-glucoside, luteolin-7-*O*-D-glucoside and chrysoeriol-7-*O*-D-glucoside. Experiments are now in progress to isolate the other active substance(s) from polyphenol fractions as well as three flavonoid glucosides.

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